FACULTEIT DIERGENEESKUNDE

VAKGROEP VIROLOGIE, PARASITOLOGIE EN IMMUNOLOGIE
LABORATORIUM VOOR IMMUNOLOGIE VAN DE HUISDIEREN

Development of immunodetection assays for penicillins and sulfonamides in food of animal origin

Patricia Cliquet

Proefschrift voorgelegd aan de Faculteit Diergeneeskunde tot het verkrijgen van de graad van Doctor in de Diergeneeskundige Wetenschappen

Promotoren: Prof. Dr. E. Cox en Prof. Dr. B. Goddeeris
Merelbeke, 2004

Dankwoord

Wie had ooit durven denken dat dit werk eindelijk voltooid zou zijn!? Ik heb er inderdaad mijn tijd voor genomen…. Dit werk is zeker niet alleen door mij tot stand gekomen. Daarom, en naar goede gewoonte, is het nu het moment om mijn dank te betuigen aan al wie van ver of van dichtbij bijgedragen heeft tot dit onderzoek.

Mijn promotoren, Prof. Dr. E. Cox en Prof. Dr. B. Goddeeris, bedankt voor de begeleiding, advies, steun en vooral, bedankt dat jullie erin bleven geloven.

Een woord van dank gaat zeker ook uit naar het voormalig Ministerie van Middenstand en landbouw, het Instituut voor Veterinaire Keuring en naar de firma Laméris. Zonder hun financiële bijdragen was dit onderzoek nooit tot stand gekomen.

Volgende personen verdienen eveneens een woord van dank:

- de leden van de begeleidingscommissie van het project voor hun kritische en opbouwende kritiek die zij wisten aan de dag te leggen tijdens de jaarlijkse bijeenkomsten, in het bijzonder Prof. Dr. D. Portetelle, Dr. K. Dierick, Dr. Ir. J. Mast en Prof. Dr. G. Maghuin-Rogister. De leden van de doctoraatsbegeleidingscommissie voor het geven van de toelating tot het indienen van dit proefschrift.

- Dr. L. Okerman, Dr. ir. K. De Wasch, Dr. S. De Baere en Prof. Dr. S. Croubels voor de analyse van stalen en het uitwisselen van ideeën. Prof. Dr. E. Schacht voor advies over de ontwikkeling van hapteen-eiwitconjugaten en uitvoering van de synthese van sulfonamiden, Dr. W. Haasnoot voor het uitwisselen van ideeën over de productie van sulfonamiden-specifieke antistoffen en K. Bonroy voor het uitvoeren van laatste testjes op de biosensor

- Dr. H. Beernaert, Dr. H. Van Oyen en F. Claeys van het WIV, voor hun steun en begrip om dit werk af te maken.

Natuurlijk bedank ik ook mijn collega's van het Labo Immunologie. Vooral Els, met de gouden handen. Vele experimenten zijn geslaagd dankzij de nauwkeurigheid waarmee je ze uitgevoerd hebt. Denise, vooral dankzij jou was er orde in het labo. Voor mij ben je echt onmisbaar voor de goede gang van zaken in het labo. Tine, Sabine en Veerle, we hebben veel gebabbeld en gelachen,…. Yves, Frank, Tanya, Annie, Timo, Annelies, Vesna, Petra, Hilde, Griet en Mieke, de leuke sfeer in het labo heeft dit werk zeker positief beïnvloed. De stagiairs en thesisstudenten Sabine, Annelies, Inge, Jeroen, Geert, Sophie die aan dit onderzoek meegewerkt hebben. Renaat, Pieter, Julien, Magda, bedankt voor het zorg dragen van de muizen en konijnen. De parasitologen, bedankt voor de leuke pauzes. Het waren best gezellige tijden, daar in Merelbeke!

Op het thuisfront wil ik mijn ouders, familie en vrienden extra bedanken voor hun onvoorwaardelijke steun en toeverlaat, en vooral voor het bezorgen van fijne ontspannende momenten.

En als laatste in de rij, maar daarom ook de belangrijkste persoon in mijn leven. Of liever, de belangrijkste personen. Want ze zijn met z'n tweeën. Claude en Sam, mijn schatten, bedankt!

Patricia

Table of contents

List of abbreviations

6-APA	6-aminopenicillanic acid
Ab	antibody
ABTS	2,2'-azino-di-(3-ethylbenzoathiazoline-6-sulfonate)
Ag	antigen
amox	amoxicillin
amox-bio	biotinylated amoxicillin
amp	ampicillin
amp-bio	biotinylated ampicillin
antibody ciELISA	competitive inhibition ELISA coated with antibody
antigen ciELISA	competitive inhibition ELISA coated with antigen
azocasein	sulfanilamide-casein diazotation conjugate
BCA	bicinchoninic acid
bgg	bovine gamma globulin
bio	biotin
bpg	benzylpenicillin or penicillin G
bsa	bovine serum albumin
$CC\alpha$	decision limit
$CC\beta$	detection capability
C_H	constant domain heavy chain
ciELISA	competitive inhibition ELISA
C_L	constant domain light chain
clox	cloxacillin
CV	coefficient of variation
DHPPP	7,8-dihydro-6-hydroxymethylpterin-pytophosphate
DHPS	dihydropteroate synthase
diclox	dicloxacillin
DMEM	Dulbecco's Modified Eagle's Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiïmide
ELISA	Enzyme-Linked ImmunoSorbent Assay
FCA	Freund's Complete Adjuvant
FCS	foetal calf serum
FIA	Freund's Incomplete Adjuvant
H	heavy chain
HAT	Hypoxanthine Aminopterin Thymidine
HBS	hepes buffered saline
HGPRT	hypoxanthine-guanosyl-fosforibosyl-transferase
HPLC	High Pressure Liquid Chromatography
HRP	horseradish peroxidase (mierikswortelperoxidase)
HT	Hypoxanthine Thymidine
IC50	inhibitory concentration at 50 % inhibition
IFP	footpad immunization
Ig	immunoglobulin
IP	intraperitoneal
IV	intravenous
K	affinity constant

k_a	association constant
k_d	dissociation constant
klh	keyhole limpet hemocyanin
L	light chain
LC-MS/MS	liquid chromatography- tandem mass spectroscopy
LOD	limit of detection
lph	limulus polyphemus hemolymph
mAb	monoclonal antibody
MBS	3-maleimidobenzoic-*N*-hydroxysuccinimide ester
MEDC	1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate
MIC	minimal inhibitory concentration
mIL-6	mouse interleukin-6
MRL	Maximum Residue Level
MW	molecular weight
NBKT of BKT	(New) Belgian Kidney Test
ND	not determined
NHS	N-hydroxysuccinimide
NT	not tested
OD	optical density
OPI	OPI media supplement
ova	ovalbumin
oxa	oxacilline
pAb	polyclonal antibodies
PABA	para-aminobenzoic acid
PBP	penicillin binding protein
PBS	phosphate buffered saline
PEG	polyethyleenglycol
ppb	parts per billion
PS	N1-[4-methyl-5-[2-(4-carboxyethyl-1-hydroxyfenyl)]-azo-2-pyridyl]sulfanilamide
psa	porcine serum albumin
PS-bio	biotinylated PS-sulfonamide
PS-ciELISA	ciELISA coated with PS-sulfonamide
PS-klh	PS-sulfonamide conjugated to klh
PS-ova	PS-sulfonamide conjugated to ova
r	correlation coefficient
RIA	radioimmunoassay
RT	room temperature
S	N-sulfanyl-4-aminobenzoic acid
S-albumin	S-sulfonamide conjugated to an albumin
SAMSA	*s*-acetylmercaptosuccinic anhydride
SD	standard deviation
Smt-bgg	sulfamethazine-bgg conjugate
Sulfa-bsa	sulfanilamide-bsa diazotation conjugate
Sulfa-glut-bsa (1)	sulfanilamide-bsa glutaraldehyde conjugate, method Van Regenmortel
Sulfa-glut-bsa (2)	sulfanilamide-bsa glutaraldehyde conjugate, method Märtlbauer
SulfaMBSova	sulfanilamide conjugated to ovalbumin using MBS as cross-linker
thyro	thyroglobulin
TLC	thin layer chromatography

TMB	3,3', 5,5'-tetramethylbenzidine
TS	N1-[4-(carboxymethyl)-2-thiazolyl]-sulfanilamide
TS-bio	biotinylated TS-sulfonamide
TS-ciELISA	ciELISA coated with TS-sulfonamide
TS-klh	TS-sulfonamide conjugated to klh
TS-ova	TS-sulfonamide conjugated to ova
V_H	variable domain heavy chain
V_L	variable domain light chain

Part I

Introduction

Introduction

During the last decades, the use of antimicrobial drugs, growth promoting agents and food additives in animal husbandry has increased considerably. As a result, residues of these drugs occur in food products derived from treated animals or reach soil and ground water after excretion via faeces and urine (Dupont and Steele, 1987; Franco et al., 1990).

These residues can be harmful for human and animal health (Milhaud and Person, 1981; Dupont and Steele, 1987), due to direct toxicity when high concentrations are present (Schmid Von, 1983), the development of antibiotic resistant pathogenic bacteria (Al-Sam et al., 1993; Anonymous, 1997b; Schwarz and Chaslus-Dancla, 2001), the disturbance of the intestinal flora (Okerman, 1995), and finally, the potential of some residues to induce hypersensitivity reactions (Burgat-Sacaze, 1981; Allison, 1985; Dewdney et al., 1991).

The presence of residues in food producing animals induces financial losses. Carcasses and products containing violating levels of residues are confiscated and the farm owner is sanctioned with extra controls (Anonymous, 1997a). Residues in milk and meat affect the fermentation process of yoghurt, cheese and sausage preparation (Mourot and Loussouarn, 1981; Allison, 1985; Koenen-Dierick and Van Hoof, 1988; Grunwald and Petz, 2003).

To protect consumers from risks related to drug residues, maximum residue levels (MRL) and withdrawal times were determined for drugs in food (Anonymous, 1990), and the inspection of food derived from treated animals was regulated (Anonymous, 1996a). Food is considered safe for consumption when no drug residue is present above the MRL. Each member of the European Union has a monitoring program to test for the presence of legal and illegal veterinary drugs in edible tissues.

During the monitoring for the presence of drug residues, a large number of samples are screened using receptor assays, immunological or microbiological methods (Allison, 1985; Charm and Chi, 1988; Kavanagh, 1989; Moats, 1990). Because a large number of samples is analysed, the ideal screening test is cheap, fast, requires no or little sample preparation and has a high sensitivity. The screening of samples will provide a majority of negative results, indicating that the samples are “compliant” with the legislation. Compliant samples require no further analysis. A minority of samples will be tested positive during

screening, indicating that the samples are suspected to be non-compliant with the legislation. In the case of a suspected non-compliant sample, this result must be confirmed by a confirmatory method (liquid or gas chromatography combined with spectrometric detection; Anonymous, 2002). The results of such analysis are considered to be exact and very reliable. The drawback of the method is the intensive sample preparation and the expensive price. It is therefore important to use screening assays with high specificity to avoid the superfluous analysis of false non-compliant samples with the expensive and laborious confirmation methods.

Actually, the Belgian residue control program for meat and meat products is currently operating in three steps. First, the kidney and sometimes muscle tissues collected at slaughterhouse are analysed in the New Belgian Kidney Test (NBKT; Anonymous, 1995) for the presence of antibiotics and other anti-microbiological substances (pre-screening). The NBKT is a microbiological assay based on the growth inhibition of bacteria on an agar plate in presence of a meat sample (Okerman, 1995; Okerman et al., 1999). The assay only indicates the presence of an anti-microbiological agent and does not discriminate between different families of agents. Therefore, suspected non-compliant samples are further screened using other microbiological assays or using immunoassays to identify the inhibitory substance or the group to which it belongs (screening). Finally, the analyte present is identified and quantified using a confirmatory method (confirmation).

Penicillins and sulfonamides are widely used in veterinary medicine. The NBKT used in the pre-screening step, can also be applied to screen samples for the presence of penicillins (Okerman, 1995). Therefore, the samples are incubated with and without penicillinase. The inhibitory activity of penicillin on the bacterial growth of the test will be abolished by penicillinase. Other assays used for screening purposes are the Penzyme Test (Everest et al., 1993) and the microbiological receptor test Charm II (Charm and Chi, 1988). These three methods detect only the microbiological active form of penicillin and not the allergenic metabolites. Penicillins are easily broken down during storage or sample preparation (Boison, 1995). As a result, an underestimation of the real penicillin concentration will occur. The screening for sulfonamides is also a problem. The NBKT is not sensitive enough (Okerman, 1995). A lot of ELISAs are commercially available, each of them highly specific for one or two sulfonamides. Thus, screening with these ELISAs would be very expensive. The Charm II Test is sensitive enough to detect the sulfonamides at the MRL, but the assay is based on

radioactivity. The penicillins as well as the sulfonamides can be identified and quantified at concentrations below the MRL using liquid chromatography coupled to spectrometry (Boison, 1995; Ito et al., 2000; Van Eeckhout et al., 2000). But these techniques can only be applied for confirmation because they are too expensive and labour-intensive for screening.

There is a need for adequate, sensitive screening tests for penicilllins and sulfonamides enabling the group-specific detection of both families. Immunochemical methods like Enzyme-Linked Immunosorbent assays (ELISA) and optical immunobiosensors are therefore interestingly because they can be designed to be specific for one group of antibiotics or chemotherapeutics, allowing the group-specific identification during the screening step of the residue control program. ELISAs are quick, sensitive and have the additional advantage of analysing several samples simultaneously (Paraf and Peltré, 1991). Optical biosensors like the Biacore™ biosensor, based on the antigen-antibody interaction can provide fast, automated, reliable and sensitive analysis (Elliot, 2001). A test kit for the group-specific detection of all sulfonamides in porcine tissues using the Biacore biosensor is nowadays available (McGrath et al, 2004).

Chapter 1

Principles of immunoassays

Chapter 1: Principles of immunoassays

1.1. Antibody and antigen

Immunochemical methods are based on the extraordinary discriminatory power of antibodies, based on the ability of the immune system of vertebrates to produce an unlimited variety of proteins (antibodies), each with an affinity for a specific foreign compound (antigen or hapten). An understanding of the physicochemical and mathematical background of the interaction between antigen and antibody, and the way it is influenced by external factors is of crucial importance for the quality of the immunoassays.

1.1.1. The antibody – antigen interaction

The binding between an antibody and an antigen is non-covalent and reversible. The interaction is the result of a combination of attractive and repulsive forces (reviewed by Van Oss, 1994). Four types of forces promote the binding: the electrostatic interaction, dispersion forces, hydrogen bonds and hydrophobic interactions. A lack of complementarity (steric factor) between the antigen and the antibody binding sites is important among the repulsive forces and prevents a close approach of the two molecules necessary for the weak, attractive forces to be effective. The major driving forces for the antigen-antibody reaction are the hydrophobic interactions. They are based on the repulsion of water by non-polar groups rather than on attraction of molecules (Tanford, 1978). The water molecules are squeezed from the binding sites, enhancing the tightness of electrostatic or ionic binding, since water molecules no longer compete with the latter. The dispersion or van der Waals forces are responsible for the attractive interaction between non-polar residues of the antigen and the antibody. They act over small distances only (the forces decrease with the inverse 7^{th} power of the intermolecular distance until a certain minimum distance) and are characterized by their additivity; the force between two large sites equals the sum of all interactions (Tijssen, 1985). The electrostatic interactions are usually not dominant in the antibody-antigen complexes. These forces are inversely proportional to the second power of the intermolecular distance and to the dielectric constant, which decreases drastically when water molecules are squeezed out. Therefore, complementarity around the bond, which determines the degree of water elimination, is directly related to energy gain. The level of ionisation depends on the pH of the immediate environment (Tijssen, 1985). Hydrogen bonding is primarily exothermic and increases in

strength by reduced temperature. Consequently, antigen-antibody interactions for which hydrogen bonding is important are more stable at lower temperature ("cold antibodies", Tijssen, 1985) (Van Oss, 1994).

The equilibrium reaction between antibody (Ab) and antigen (Ag) may be expressed as:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab\text{-}Ag$$

Where k_a and k_d represent the association and dissociation rate constant. The equilibrium (affinity) constant K may be established according to the law of mass:

$$k_a[Ab][Ag] = k_d[Ab\text{-}Ag] \text{ and } K = k_a/k_d = [Ab\text{-}Ag]/[Ab][Ag]$$

with [Ab], [Ag] and [Ab-Ag] the equilibrium concentration of the free antibody, free antigen and the antibody-antigen complex, respectively (Van Regenmortel and Azimzadeh, 1994). K is the equilibrium or affinity constant (M^{-1}) and is a parameter for the closeness of fit between antigen and antibody or the complementarity between the binding site of antigen and antibody. The association rate constants k_a are very similar for various antibody-antigen systems, i.e., for many haptens around 10^7-10^8, which are only slightly below the diffusion rate and depend directly on, but cannot be faster than, the diffusion controlled encounter of an antigen with its antibody. The initial rate for protein antigens can be about 100 times lower, due to their slower diffusion. In contrast, k_d can vary from 10^{-4} for high affinity antibodies to 10^3 for low affinity antibodies, depending on the closeness of fit (Tijssen, 1985). An antibody of extremely good fit can have an affinity around 10^{11} M^{-1}, K-values around 10^9 M^{-1} are fairly normal, while low affinity antibodies will display values below 10^6 M^{-1} (Van Regenmortel and Azimzadeh, 1994).

1.1.2. The antibody

Antibodies or immunoglobulins are proteins synthesized by B-lymphocytes of an animal or human in response tot the presence of a foreign substance. The basic structure of an intact immunoglobulin is a Y-shaped molecule composed of two heavy (H) and two light (L) chains. The H-chain (50-70 kDa) is built out of one variable domain V_H and three constant C domains C_{H1}, C_{H2} and C_{H3}. The L-chain (about 25 kDa) consists of a V_L and a C_L domain. All

these domains consist of about 100 amino acid residues, are homologous in their primary structure, and are independent, stable structural units (Stryer, 1988). The polypeptide chains between the domains are susceptible to protease cleavage. The immunoglobulin molecule is cleaved by pepsin in the hinge region, linking the two arms to the base of the molecule. The two arms form together the $(F_{ab})_2$ fragment, while the base of the molecule is called the F_c fragment. After cleavage of the bivalent F_{ab} fragment by papain, the two arms are released from each other and are known as F_{ab} fragments (Tijssen, 1985).

Antibodies have two functions: recognition of the antigen and activation of the immune system. The first function is mediated by the antigen-binding sites, situated between the V_L and V_H domain, at the top of the F_{ab} fragments and known as complementarity determining regions (CDR). The activation of the protective system depends on the different heavy chains. Immunoglobulins are therefore classified according to the structure of the heavy chain into IgG, IgM, IgA, IgE and IgD. According to the animal species, some classes are subdivided into subclasses, designated by addition of a number (IgG_1, IgG_2,…). Table 1.1 shows some characteristics of the human immunoglobulins. IgG is involved in secondary responses, placental transfer and complement fixation; IgA is the major class of antibodies in external secretions (saliva, tears, bronchial and intestinal mucus). IgM is produced in primary responses and in response to T-cel independent antigens. IgM is also a powerful agglutinin and is important for complement fixation. IgD is an early receptor on lymphocytes and IgE plays a role in allergic reactions (Tijssen, 1985; Stryer, 1988).

Antibodies are synthesized against all antigenic conformations the immune system could possibly encounter (Creighton, 1993). Each antibody-producing cell produces a single antibody molecule, expressed as membrane protein (B-cell receptor). When such a cell encounters an antigen that is recognized by its receptor, the cell is stimulated to undergo cell division, to proliferate and to produce large quantities of antibodies. This means that an enormous number of antibodies with different antigen specificity are produced. This diversity is generated by a special mechanism during the biosynthesis of the antibodies. The variable domains are encoded by separated gene segments: variable (V_H), diversity (D) and joining (J) for the heavy chain; V_L and J_L for the light chain. Different antibodies are generated by joining these segments in different combination. Further genetic variation is introduced at the sites where the segments are joined together by the genetic fusion mechanism. Most of the

variations occur in the residues comprising the three CDR regions of each polypeptide chain (Creighton, 1993).

Table 1.1.: Characteristics of the human immunoglobulins (from Tijssen, 1985).

	IgG	IgA	IgM	IgD	IgE
Heavy Chain	γ	α	μ	δ	ε
Mass H chain (kDa)	50	65	70	70	72.5
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Mass L chain (kDa)	25	25	25	25	25
Secretory component	No	yes	no	no	no
Polymer	monomer	dimer	pentamer	monomer	monomer
Valence for Ag binding	2	2 or 4	10	2	2
Approximate concentration in serum (g/l)	10	2	1	0.05	10^{-4}
Mass (kDa)	150	160-400	900	185	200

The lymphocyte precursor cell will first express IgM on its surface, followed by IgD. After stimulation by contact with an antigen, the cell will differentiate into memory cells and antibody producing plasma cells. During the primary response, IgM is produced. After a second contact with the antigen, mostly antibodies of the IgG class, but also some IgE and IgA are then formed (Tijssen, 1985). During this process of repeated contact with the antigen, the genetic segments coding for the variable region of the antibody molecule undergo mutations at a rate much higher than normal. Cells producing antibodies with higher affinity are then selected. Consequently, the immune response to an antigen changes in time. Initially, low affinity antibodies are produced ($K_d = 10^{-5}$- 10^{-7}), but with time antibodies of increasing affinity are produced (Creighton, 1993).

1.1.3. The nature of immunogens, antigens and haptens

Two fundamental requirements must be fulfilled by a molecule to be immunogenic: 1) it should be foreign to activate the immune system, and 2) it must be of a certain complexity to react with the different compounds of the immune system. Immunogenicity is thus the

ability to stimulate a specific response, here the production of specific antibodies. Only restricted portions of a macromolecule are involved in the antigen-antibody interaction. These regions are called epitopes and are rather small (5-7 amino acids). Epitopes have to fit in the antigen binding sites (paratopes) on antibodies. Macromolecules may contain many epitopes on one molecule. Antigens carry a number of epitopes. Immunogens carry in addition to these epitopes, T-cell epitopes that play a role in the immune response. For the induction of antibody production, both a B-cell and a T-cell immune response are required. The precursor of each antibody-producing lymphocyte makes Ig of only one specificity, which is expressed as membrane protein. When a specific immunogen (peptide) binds to this surface antibody, the cell will proliferate and differentiate into specific-antibody secreting cells and into a clone of memory cells (responsible for the faster and stronger secondary response). This proliferation however, will only occur with some help from T-cells triggered by the immunogen. Foreign proteins are phagocytized and degraded into peptides by antigen presenting cells (macrophages, monocytes, dendritic cells, B-cells). The peptides are presented at the surface of the antigen presenting cells and can now be recognized by T-cell receptors. Once recognized, the T-cell will proliferate and differentiate into T-helper cells and produce cytokines necessary for the B-cell to proliferate and differentiate (Stryer, 1988; Jemmerson, 1995).

Haptens are antigens but not immunogens because they lack the carrier determinant or T-cell epitope. Haptens can be rendered immunogenic after covalent attachment to a suitable immunogenic carrier protein (Van Regenmortel et al., 1988). Most of the time haptens are small molecules, for example antibiotics or toxins, but immunogenicity and not size is the criterium. Large non-immunogenic molecules can also act as haptens and rendered immunogenic after conjugation to a suitable carrier. An antigen must also be degradable to be immunogenic. For example, the weak immunogenicity of D-amino-acids polymers could possibly be due to the lack of processing by macrophages (Tijssen, 1985). Haptens are not only coupled to carrier proteins for immunogenic purposes. Conjugates are also useful as reagents for the detection of antigen by immunoassays (Van Regenmortel et al., 1988).

A variety of methods for covalent coupling exist. These methods may employ a functional group on a terminal residue or on internal residues as sites of attachment. The reactive groups on haptens are also frequently their specific immunodeterminant, which distinguishes the hapten from related molecules. The use of these groups can thus decrease

the specificity of the generated antibodies (Murphy, 1980; Tijssen, 1985). Although in some situations the recognition of a wider range of haptens is desirable (for example the group-specific detection of penicillins), specificity is often required. Superior specificity can be obtained by choosing a group common to related molecules as the site of linkage (Cook et al, 1976). For example, antibodies induced with sulfonamides linked at the aromatic amino group to carrier proteins were highly specific for that linked sulfonamide and showed no or very little cross-reactivity for other sulfonamides (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Sheth and Sporns, 1990; Muldoon et al., 2000; Lee et al., 2001; Spinks et al., 2001). The linker molecule can be homobifunctional, with the same reactive group at each end, or heterobifunctional, with two different reactive groups. The length of the linker molecule can be long or short, with an aliphatic or aromatic character (Kirkley et al, 2001). Several studies analysing the influence of the linker molecule on the immunogenicity of a hapten-carrier conjugate or specificity of the immunoassay using a conjugate suggest that the selection of the linker molecule depends on the hapten and should be evaluated on a case-by-case basis (Kirkley et al., 2001). The choice of the carrier is also important. The most common carriers are serum albumin of various species, keyhole limpet hemocyanin, thyroglobulin or fibrinogen (Tijssen, 1985). The coupling rate depends on the linker molecule and the reagents ratio. For immunization, a certain number of 8 to 25 haptens per carrier molecule is optimal, but that may be quite different for immunoassay application (Tijssen, 1985; Van Regenmortel et al., 1988). Too many hapten molecules per carrier can lead to tolerance whereas a lack of immune response may be observed using conjugates with a low hapten load. Landsteiner (1945) observed that the antibody response is mostly directed against the group of the hapten the farthest away from the linkage. Otherwise, antibodies against the linker molecule are often generated (Eisen and Siskind, 1964). It is therefore important to use different linker molecules in conjugates used in the immunizations than in immunoassays (Van Weemen and Schuurs, 1975).

1.1.4. Production of polyclonal antibodies

Polyclonal antibodies are obtained by immunizing animals and collecting the serum. The immunization efficiency may be influenced by many factors related to the immunogen, the animal and the immune response. As mentioned before, an antigen will only stimulate antibody production if it is immunogenic: it must be a foreign, degradable molecule with a certain complexity. Moreover, the purity of the immunogen is important for polyclonal

antibody production since traces of very immunogenic impurities can overwhelm the principle antigen response (Booman, 1988).

The response to weak immunogens can be enhanced using an adjuvant. Adjuvants are immunopotentiators that enhance the activation of the immune system resulting in an increased immune response (Boersma and Claassen, 1995). Adjuvants like aluminum salts or mineral oils, change the physical state of water-soluble immunogens by forming depots so lowering the rate of elimination (Nicklas, 1992). This prolonged persistence of the immunogen in tissues results in a continuous stimulation of the immune system. Some adjuvants (endotoxin) increase protein synthesis. Other adjuvants stimulate different cellular parts of the immune system: mycobacteria expand the T-cell population, endotoxin and *Bordetella pertussis* stimulate B-cells, and many adjuvants of bacterial origin mobilize macrophages. Complete Freund's adjuvant causes local formation of granulomas that are rich in macrophages and immunocompetent cells. Freund's incomplete adjuvant is a mineral oil with a stabilizer; complete Freund's adjuvant contains in addition heat-killed *Mycobacterium tuberculosis*. Freund's adjuvant has been used for many years and is considered to be one of the most effective adjuvants (Boersma and Claassen, 1995).

Another important factor for the induction of an immune response is the way the immunogen is administered: intramuscular, intravenous, intradermal, intraperitoneal or subcutaneous. Intramuscular, intraperitoneal and subcutaneous injections are most commonly used because they give sustained stimulation of the immune system. Intradermal inoculation is ideal for injection of small volumes and a prolonged release. Intravenous immunization is preferred for a final booster immunization since the immunogen will be mainly captured in the spleen and since no adjuvants can be used (Harlow and Lane, 1988).

The choice of animal is mostly one of convenience rather than necessity. Generally, rabbits, sheep and goats are used for polyclonal antisera production and mice and rats for monoclonal antibody production.

One important restriction of polyclonal antibodies is the lack of reproducibility. Antisera of different animals against the same immunogen and even antisera taken at different times from the same animal can vary drastically in characteristics (specificity, avidity). This problem may be overcome by the production of monoclonal antibodies or the ability of

selecting and cloning one individual antibody-producing cell. Furthermore, the methods of protein engineering make it possible to manipulate the genes encoding antibody synthesis, to express them in micro-organism and to select antibodies with the desired specificity (Kramer, 2002).

1.1.5. Production of monoclonal antibodies

Lymphocytes do not have the ability to grow *in vitro*. This problem can be overcome by transformation of the B-cells *in vitro* using oncogenic viruses or by fusion of the antibody producing cells with myeloma cells.

The discovery of transformation of human B-lymphocytes with Epstein Barr virus was very promising since high percentages of the transformed cells were obtained. Rabbit cells could also successfully be transformed using SV40, but the method was not found suitable for murine cells (Zurawski et al., 1978; Steinitz et al., 1980).

In 1975, Cesar Milstein and Georges Köhler discovered that monoclonal antibodies can be obtained by fusion of an antibody producing cell with a myeloma cell. Myelomas are uncontrolled dividing B-lymphocytes. Tijssen (1985) reviewed different strategies to produce monoclonal antibodies. A mouse is immunized with a given antigen, and its spleen is removed after several days. The lymphocytes are fused with myeloma cells by exposing them to polyethylene glycol, a polymer that induces cell fusion (Harlow and Lane, 1988). The fusion can also be stimulated using electric pulses (Karsten et al., 1988; Van Duijn et al., 1989). The myeloma cell line lacking hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) is used to enable the selection of hybrids. The enzyme HGPRT catalyzes the synthesis of inosinate (a precursor of AMP and GMP) in the salvage pathway of nucleotide synthesis. The cells are grown in a medium containing hypoxanthine, aminopterin and thymine (called HAT medium) to kill unfused myeloma cells. The role of aminopterin in this medium is to block the de novo synthesis of nucleotides. Unfused myeloma cells cannot use hypoxanthine because they lack HGPRT. Spleen cells contain HGPRT, but they die in cell culture because they are not able to proliferate *in vitro*. Hybrid cells, called hybridoma cells, survive *in vitro* because they obtained the ability to proliferate from their myeloma cell parent and the HGPRT gene from the lymphocyte cell parent (Tijssen, 1985; Stryer, 1988).

Hybridoma cells are grown in wells in tissue-culture plates. The supernatants from these wells are screened for the presence of specific antibodies. The cells of positive wells are cloned and screened to obtain hybridomas of a single kind. The hybridomas can be frozen and stored for long periods (Harlow and Lane, 1988).

1.1.6. Monoclonal *vs* polyclonal in immunoassays

Antisera taken at different points of time from the same animal can vary in their properties. In contrast, monoclonal antibodies are produced by a single clone of B cells and, consequently, have the same specificity and sensitivity. The most important advantage of monoclonal antibodies is therefore the possibility to standardize assay methods. Other advantages are that they can be produced in unlimited quantities (Booman, 1988) and that pure immunogen is not required for the immunization due to cloning and selection during the production. On the other hand, monoclonal antibody production is laborious and time consuming. The number of species that can be used to produce monoclonals is limited. Mostly mice are used. Fusion of myeloma cells with cells of other species leads to rapid segregation of chromosomes (Yarmush et al., 1980). Each monoclonal antibody may have very specific properties, in contrast to the average of polyclonal Ig. Monoclonal antibodies may have biological functions different from the corresponding polyclonal antisera and may be much more sensitive to inactivation by freezing and thawing, changes in pH or other physical properties (Mosmann et al., 1980) important for their purification (Tijssen, 1985).

The strategy of the immune system to produce polyclonal antibodies results in two important bonus effects: the affinity bonus and the specificity bonus. Both are eliminated by cloning. Most monoclonal antibodies have affinities far below the corresponding conventional antisera (Booman, 1988; Van Oss, 1994). The specificity of a monoclonal is also sometimes lower than expected. Some may cross-react. In contrast to polyclonal antibodies, this cross-reaction cannot be removed with immunosorbents. A monoclonal cannot distinguish between different antigens if these antigens have the same epitope (Tijssen, 1985). This characteristic however, makes the application of monoclonal antibodies in immunoassay for the group-specific detection of antibiotics interesting.

The affinity is a thermodynamic measurement of the strength of the non-covalent interactions between one site of the antibody and of the antigen. In contrast, the avidity is an

operational term expressing the ability of an antiserum to bind antigens and therefore depends not only on affinity but also on multivalency of the antibody and other non-specific factors. The interaction between antibody and antigen is a continuous process of association and dissociation during which the two molecules may become separated. In the case of multivalency, the multiple bounds do not separate at the same moment, making it less likely that the complex becomes separated. For example, the avidity of the multivalent IgM is 10^2-10^4 times higher than the affinity of the individual F_{ab} fragments (Van Oss, 1994).

Avidity is thus an important characteristic of polyclonal antisera, since they generally contain antibodies against all determinants of an antigen, in contrast to monoclonal antibodies.

1.2. Immunochemical methods

1.2.1. The Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassays (EIA) were developed in the mid-sixties for the identification and localization of antigens in histological preparations and for the identification of antigens precipitated in immunodiffusion and –electrophoresis experiments (Nakane and Pierce, 1966; Avrameas and Uriel, 1966). These enzyme immunohistochemistry methods were found very useful in other fields. The observation that antigens or antibodies can be immobilized (coated) on solid phases and the labelling of the immunoreactants with an enzyme (Rubenstein et al., 1972) made it possible to apply similar methods for the quantification of immunoreactants in test tubes. The development of methods to produce monoclonal antibodies (Kohler and Milstein, 1975) enhanced the possibility of standardization of EIA with higher specificity and sensitivity, and contributed to new assay designs. Solid phase enzyme immunoassays are still one of the most applied test systems. The great advantage of these assays is the simplicity, their sensitivity and specificity, and the ability of analysing a lot of samples simultaneously (Paraf and Peltré, 1991). Immunoassays are widely used in human and veterinary medicine diagnostics, environmental and forensic investigations, and food monitoring (Paraf and Peltré, 1991).

Immunoassays can be divided into homogeneous and heterogeneous, competitive and non-competitive, direct and indirect assays.

In the non-competitive homogeneous enzyme immunoassays, all reactants remain in solution during the test. There is no separation of labelled and free reactant before signal generation. The distinction between bound and free conjugate is achieved by labelling two monoclonal antibodies, each specific for a different epitope of the antigen, with two different enzymes selected so that one produces the substrate for the other (e.g. glucose oxidase and peroxidase). The enzyme activity is only detected when the two labelled antibodies react with the antigen, bringing their labels close to each other (Tijssen, 1985).

An example of a competitive homogeneous enzyme immunoassay is the enzyme multiplied immunoassay technique (EMIT). In this assay, the hapten is detected using an enzyme-hapten conjugate. The activity of the conjugated enzyme is modulated by the reaction of an anti-hapten antibody with the haptenated enzyme, either by steric hindrance or by changes in configuration of the enzyme. Competing free hapten will decrease this modulation, so increasing the signal (Tijssen, 1985).

In the heterogeneous assays, the reacted and unreacted components are separated before signal generation. Therefore, one of the reactants is immobilized on a solid phase (plastic, membrane, latex,…), the sample is added, the unreacted components are removed by washing, and finally the antibody-antigen complexes are visualized using an enzyme induced signal generation. In the direct assay, the antibody or antigen is directly labelled with an enzyme. In the indirect assay, a labelled anti-Ig antibody is used to detect the bound antibody (Tijssen, 1985). An important advantage of a solid phase assay compared to the homogeneous one, is the removal of interfering components during washing steps. Furthermore, solid phase assays are far more sensitive than the homogeneous assays (Delagneau and Masseyeff, 1990; Van Peteghem and Daeseleire, 2003).

Heterogeneous solid phase enzyme immunoassays are also called enzyme-linked immunosorbent assays (ELISA). Three kinds of ELISAs are currently applied for the detection of food contaminants: the sandwich ELISA, the antigen competitive ELISA and the antibody competitive ELISA.

The sandwich ELISA is a non-competitive immunoassay. The antigen in the sample is captured between an antibody immobilized on the solid phase and another antibody free in solution. Both antibodies are specific for a different epitope of the antigen. If the indirect

method is used to detect the captured antigen, the free antibody should be from a different species than the coated antibody, to prevent binding of the species-specific labelled anti-Ig antibody with the coated antibody.

The competitive ELISA (cELISA) or competitive inhibition (ci)ELISA is the most appropriated immunoassay for the detection of low molecular weight analytes in solution, like residues in meat extracts or milk (Tijssen, 1985). Because this type of ELISA will be used in the experimental part of this thesis, the ELISA is discussed in more detail.

Two kinds of ciELISA can be distinguished: the antigen ciELISA and the antibody ciELISA (Figure 1.1 and 1.2).

In the antigen ciELISA, the antigen is immobilized on the surface of the ELISA plate (Figure 1.1). Low molecular weight analytes or haptens are too small to be immobilized. In that case hapten-protein conjugates can be used. After incubation, the unbound molecules are washed away. The wash step occurs after each incubation step, except after adding the colour reagent. After coating, free space is blocked with an irrelevant molecule, like glycine, bovine serum albumin or casein. The blocking step is crucial to avoid non-specific bindings during the assay performance. Subsequently, the sample containing the antigen to be detected is added to the plate, together with an antigen-specific antibody. During the incubation, there will be competition between the antigen in solution and the antigen on the plate for binding to the specific antibody. After washing, only the antibodies bound to the coated antigen will remain. In the indirect ciELISA, these antibody-antigen complexes are detected using labelled species-specific anti-Ig polyclonal antibodies. The horseradish peroxidase (HRP) enzyme is often used as the label, but other enzymes can be used (Alkaline phosphatase, β-glucuronidase). The colour development after adding a substrate for the enzyme (together with a chromogen in case of HRP) is measured using a spectrophotometer. The intensity of the colour development is inversely related to the amount of free antigen in the sample. Maximum signal is obtained when there is no free antigen in the sample.

In the antibody ciELISA, the antigen-specific antibodies are coated on the surface of the ELISA plate (Figure 1.2). After blocking, the sample containing the free antigen, is added together with labelled antigen. Competition will occur between the unlabelled antigen in the sample and the labelled antigen for binding to the coated antibodies. Here again, the intensity

of the colour development will be inversely related to the amount of unlabelled antigen in the sample.

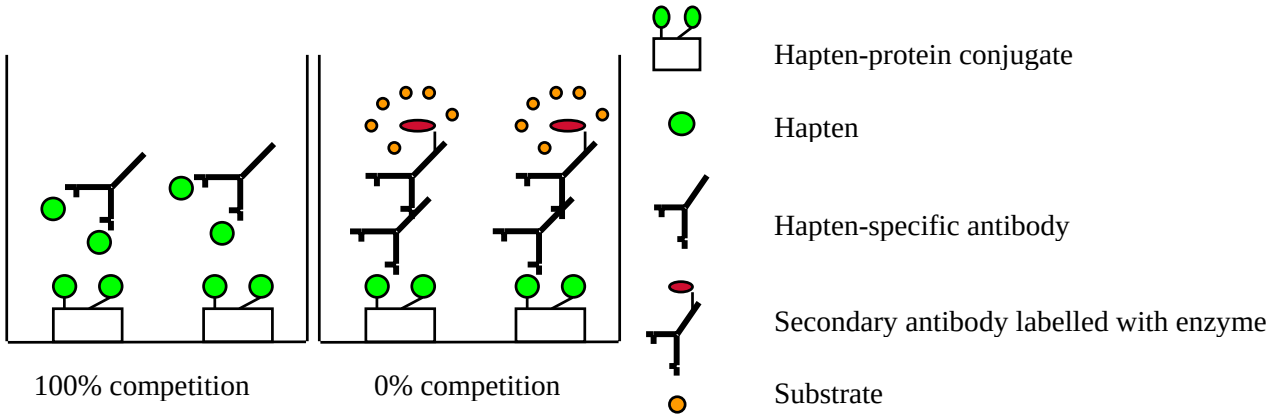


Figure 1.1: The antigen ciELISA

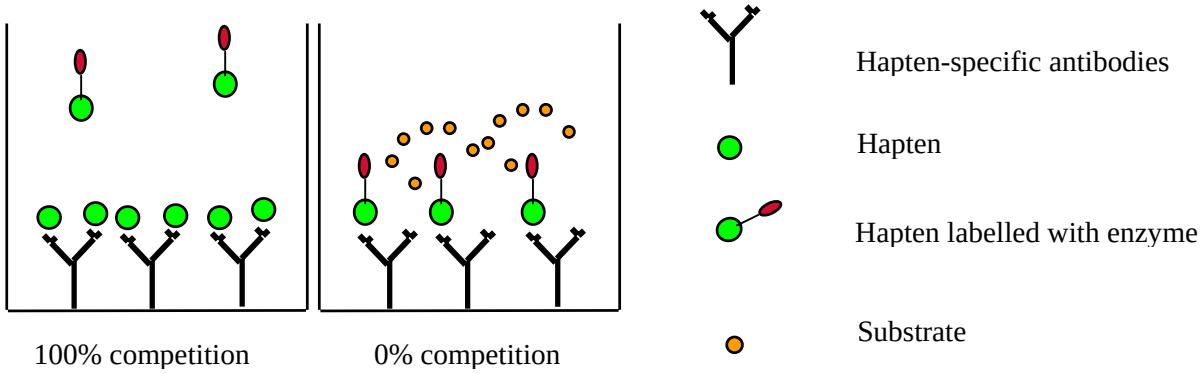


Figure 1.2.: The antibody ciELISA

Immunoassays are not only characterized by their design, but also by the label used to visualize the antigen-antibody interaction. A lot of labels exists: enzymes, radioactive, chemiluminiscent and fluorescent labels, stable free radicals, latex particles and bacteriophages (Tijssen, 1985).

Before the introduction of enzymes in immunoassays, radioimmunoassays (RIA) were mostly applied for diagnostic purposes and basic research. However, the use of radioactive agents implies important inconveniences: special expensive equipment required for the measurement of radioactivity, radioactive waste, potential health hazard, etc. Enzyme immunoassays offer the same specificities as RIA and sometimes even higher detectabilities, without the disadvantages of radioactivity (Tijssen, 1985).

Alkaline phosphatase (AP), β-galactosidase (β-GAL), and most of all horseradisch peroxidase (HRP), are commonly used labels in ELISA. A colour development will be obtained when adding the substrate for the enzyme. In case of HRP, the substrate (peroxide) is added together with a chromogen. The enzyme HRP catalyses the reaction of peroxide (H_2O_2) into H_2O and O_2, causing the oxidation of the chromogen, what finally results in a colour development. Frequently used chromogens for HRP applied in ELISA are 3,3',5,5'-tetramethylbenzidin (TMB) and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) (Tijssen, 1985).

Another detection system is based on the strong interaction between biotin, a B-vitamin and streptavidin, a protein isolated from *Streptomyces avidinii* (Chaiet and Wolf, 1964). Biotin is labelled to the antibody or antigen, streptavidin is coupled to an enzyme. The high affinity of biotin for streptavidin enables sensitive detection and low background levels. Amplification of the signal is achieved by using biotin as label for the immunoreactant and unlabelled streptavidin. Streptavidin has four binding sites for biotin. After reaction of streptavidin with the biotin coupled to the immunoreactant, there are still three binding sites available. These can be used for the binding of enzyme-labelled biotin. This allows the enzymatic activity of three enzyme molecules instead of one when enzyme-labelled streptavidin is used, and will enhance the colour development (Tijssen, 1985).

1.2.2. The BIAcore™ optical biosensor

Optical biosensors, such as BIAcore are becoming widely considered for food quality and safety control. The BIAcore technology provides fast, automated, reliable, robust and high capacity multi-residue analysis. Analysis of one sample is completed within minutes. Immunobiosensor assays for the detection of sulfonamide residues in milk, chicken sera, porcine bile and muscle tissues have been described (Sternesjo et al., 1995; Crooks et al., 1998; Elliott et al., 1999; Bjurling et al., 2000; Haasnoot et al., 2003). Test kits for clenbuterol, streptomycine, sulfadiazine, sulfamethazine, and for the group-specific detection of all sulfonamides are already commercially available.

A biosensor can be described as a device combining a biological detection system with a signal transducer. The transducer generates a measurable signal as a result of a change in

concentration of a given molecule at the detection surface. Dependent on the transducer, different biosensors were developed: optical, membrane and electrode directed amperiometric or potentiometric biosensors (Robinson, 1993).

The Biacore™ optical biosensor is based on the surface plasmon resonance (SPR) phenomenon, which allows the measurement of interactions between molecules in real time (Figure 1.3; Jonsson et al., 1991; Panayotou, 1998).

SPR detection is based on the changes of refractive index of the medium close to a thin metal layer. When plane polarized light coming from a medium with higher refractive index (prism; glass) reaches the interface with a medium with lower refractive index (in the flow channel; water), it is totally internally reflected at a certain angle of incidence. A specific component of the incident light, the evanescent wave will penetrate into the medium with lower refractive index. If the interface between the two media is coated with a thin metal film (gold in the case of Biacore™ biosensor), the evanescent wave will interact with free oscillating electrons, called plasmons, in the metal film. This phenomenon is observed as a decrease in intensity of the reflected light and occurs thus at a certain angle of incident light, called the resonance angle. In the Biacore™ biosensor, one reactant is immobilized on the sensor surface and the other is injected over the surface using a constant flow rate. Binding or dissociation at the close vicinity of the sensor surface causes changes in refractive index and thus a shift in the resonance angle, which is measured by the detection system of the biosensor. The response is expressed in resonance units (RU) and 1 RU corresponds to a shift in angle of 0.0001°, or a change in mass concentration of 1 pg/mm^2 on the sensor surface (Jonsson et al., 1991). By plotting the measured angular shift against time, a sensorgram is obtained illustrating the progress of the interaction at the sensor surface in real time (Figure 1.3).

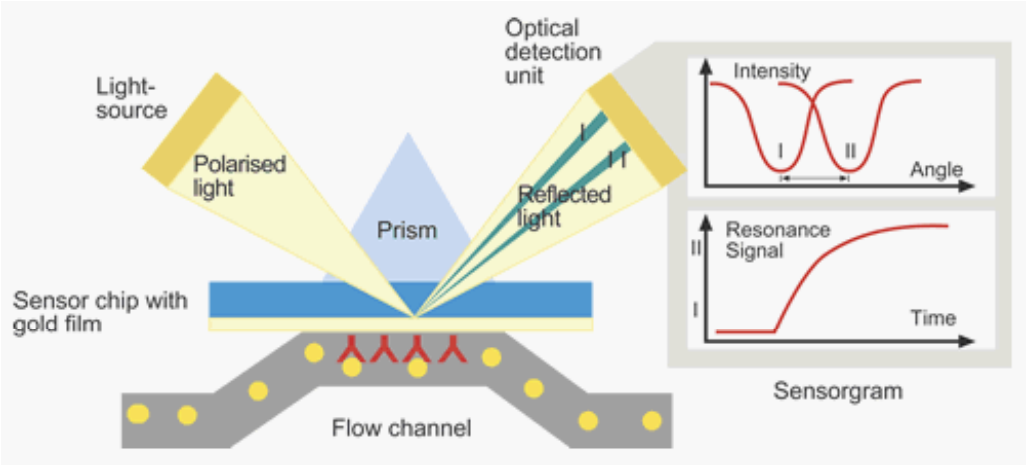


Figure 1.3: The Biacore optical biosensor detection system based on surface plasmon resonance (SPR). Binding or dissociation of molecules to the sensor surface causes changes in refractive index near the surface, resulting in a shift in the SPR angle (from I to II).

Chapter 2

Penicillins

Chapter 2. Penicillins

2.1 Introduction: use of penicillins in veterinary husbandry

In 1929, the bacteriologist A. Fleming observed that the growth of *Staphylococcus aureus* colonies was inhibited by the presence of contaminating *Penicillium notatum* colonies. This observation resulted in the discovery of one of the most remarkable therapeutic molecules, penicillins. Benzylpenicillin was first used successfully to treat infections in 1941. Benzylpenicillin is active against most Gram+ organisms and some Gram- organisms (e.g. *Actinomyces* species). Because of its instability in acidic conditions, the use of benzylpenicillin is restricted to parenteral administration. Penicillin V, another natural occurring penicillin with the same spectrum but lower activity as benzylpenicillin, is stable under acidic conditions and can therefore be orally administrated (Butaye, 2001).

Increased bacterial resistance to these natural penicillins has led to the development of more semisynthetic penicillins (Oshiro, 1999). Addition of an amino-group to the benzylpenicillin molecule resulted in the aminopenicillins, ampicillin and amoxicillin, both with broader spectrum of activity. They are both active against most aerobic Gram+ cocci and some Gram- organisms like *Salmonella, Shigella, E. coli*. Because these penicillins can be inhibited by β-lactamase activity, they are often combined with β-lactamase inhibitors (e.g. clavulanic acid). The β-lactamase resistant penicillins (oxacillin, cloxacillin, dicloxacillin) are moderately effective against pneumococci and streptococci and highly active against most staphylococci. They are frequently used in bovines for treatment and prevention of mastitis (Butaye, 2001). Another evolution in the development of new penicillins is the synthesis of molecules with activity against *Pseudomonas aeruginosa* such as ureidopenicillins (e.g. piperacillin, mezlocillin) and carboxypenicillins (e.g. carbenicillin, ticarcillin). However, these penicillins are not registered for veterinary applications. They should only be used in human medicine for the treatment of serious infections, in particular in hospitals (Butaye, 2001).

Beta-lactams are used in veterinary medicine at therapeutic levels to treat infectious diseases and sometimes also to prevent infections. They were used at sub-therapeutic levels as feed additive, to increase feed efficiency, promote growth and prevent infections (Boison, 1995). The penicillins authorised for use in veterinary medicine are shown in table 2.1 and

figure 2.1. The penicillins authorized in feed for animals are amoxicillin (300-400 mg/kg feed for pigs; 2000-16000 mg/kg feed for fish), benzylpenicillin (83 mg/kg feed for pigs) and penicillin V (200 mg/kg feed for pigs) (Anonymous, 1992; Anonymous, 2000).

The MRL-value for ampicillin, benzylpenicillin and amoxicillin is 50 ppb (µg/kg, ng/ml) in meat products and 4 ppb in milk for all food producing species, for penicillin V 25 ppb in porcine muscle, liver and kidney tissues, and for oxacillin, cloxacillin and dicloxacillin 300 ppb in meat and 30 ppb in milk for all food producing species (Anonymous, 1990).

Table 2.1.: Penicillins authorized for use in veterinary medicine.

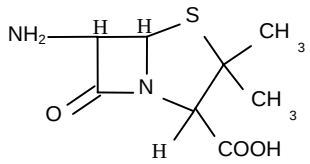
Group of Antibioticum	Penicillin
Gram+ spectrum	Benzylpenicillin (pen G), Phenoxymethyl penicillin (pen V)
Broad spectrum	Ampicillin, Amoxicillin
β-lactamase resistant penicillin Gram+ spectrum	Oxacillin, Cloxacillin, Dicloxacillin
Combination with β-lactamase inhibitor- broad spectrum	Amoxicillin + Clavulanic acid

2.2 Physicochemical and biological properties

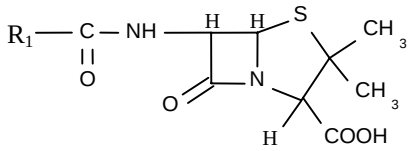
Penicillins belong to the group of β-lactam antibiotics, together with β-lactamase inhibitors and cephalosporins (Boison, 1995). The basic structure of penicillins, the 6-aminopenicillanic acid, consists of three components: a thiazolidin ring, a β-lactam ring and a side chain (Figure 2.1).

Bacterial β-lactamase hydrolyzes the β-lactam ring, resulting in the production of inactive penicilloic acid (Bush et al, 1995; Boison, 1995). Penicillins are also relatively unstable in aqueous solution. Their degradation is catalyzed both by acids and bases (Hou and Poole, 1971). The aqueous instability of β-lactams has important consequences in sample analysis, storage of a stock solution and storage of incurred tissues. It has been demonstrated that benzylpenicillin is fairly stable in solution between pH 5 and 9 (Wiese and Martin, 1989a), and in animal tissues, milk and plasma when stored at –76°C (Wiese and Martin,

1989b; Boison et al., 1992; Boison et al., 1994). Methanol rapidly degrades penicillins into their penicilloic acid esters, while 25% acetonitril or ethanol in water results in the least degradation (Boison, 1995).

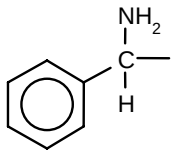


6-aminopenicillanic acid

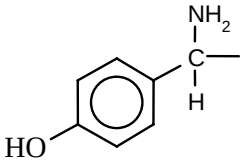


Penicillin nucleus

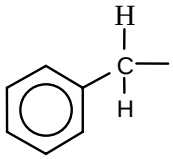
Side Chain Residue



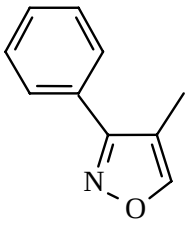
Ampicillin



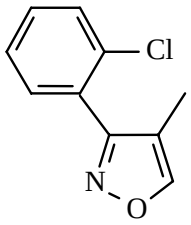
Amoxicillin



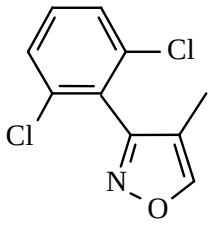
Benzylpenicillin



Oxacillin



Cloxacillin



Dicloxacillin

Figure 2.1: Chemical structure of 6-aminopenicillanic acid, of the common structure of penicillins and of the side chain residues of the tested penicillins.

The bactericidal action of penicillins includes the inhibition of cell wall synthesis and activation of endogenous autolytic mechanisms, causing cell lysis and death. The drug will first penetrate into the cell wall, which contains cross-linked peptidoglycans that maintain the structural stability of the wall. The cross-linking reaction is catalyzed by enzymes located at the inner surface of the wall. These enzymes contain penicillin-binding proteins (PBPs). Binding of penicillin on the PBPs causes termination of the peptide chain linkage and inhibits the formation of a normal peptidoglycan structure. Binding of penicillins to the PBPs also switches off endogenous inhibitors of bacterial autolysins and subsequently causes cell lysis. PBPs are species specific, and have a different affinity for different β-lactam antibiotics (Blumberg and Strominger, 1974; Spratt, 1983).

2.3. Metabolization of penicillin

Penicillins are distributed rapidly in most extracellular fluids. The concentration in the extracellular fluids is 50 to 100 % of the highest plasma concentration. Low distribution occurs in spinal fluid (Divers, 1996). Approximately 50 % of the penicillin will be bound to plasma proteins.

Penicillins undergo little metabolization and are excreted largely unchanged into the urine. Thirty to 60 % of the penicillin becomes conversed to inactive metabolites, some of them being potential allergens. Excretion of penicillins or their metabolites occurs mainly in urine and in lesser extent in bile, while very low amounts are found in milk, saliva and sweat. For benzylpenicillin, renal excretion (80 % tubular secretion, 20 % glomerular filtration) contains 40 to 70% of the active molecule and 30 to 60 % metabolites (Divers, 1996).

The β-lactam ring of penicillin spontaneously opens under physiological conditions, forming the penicilloyl group. Approximately 95 % of the penicillin molecules that irreversibly bind to proteins form penicilloyl moieties and therefore this metabolite is designated as the major determinant. Penicillin is also degraded by other metabolic pathways forming other antigenic determinants in smaller quantities. Penicilloic acid, penicillenyl, penicillenic acid and penicillamine are such minor determinants. (Dewdney et al, 1991; Chowdhury and Lieberman, 1999). The haptens derived from penicillin are shown in figure 2.2.

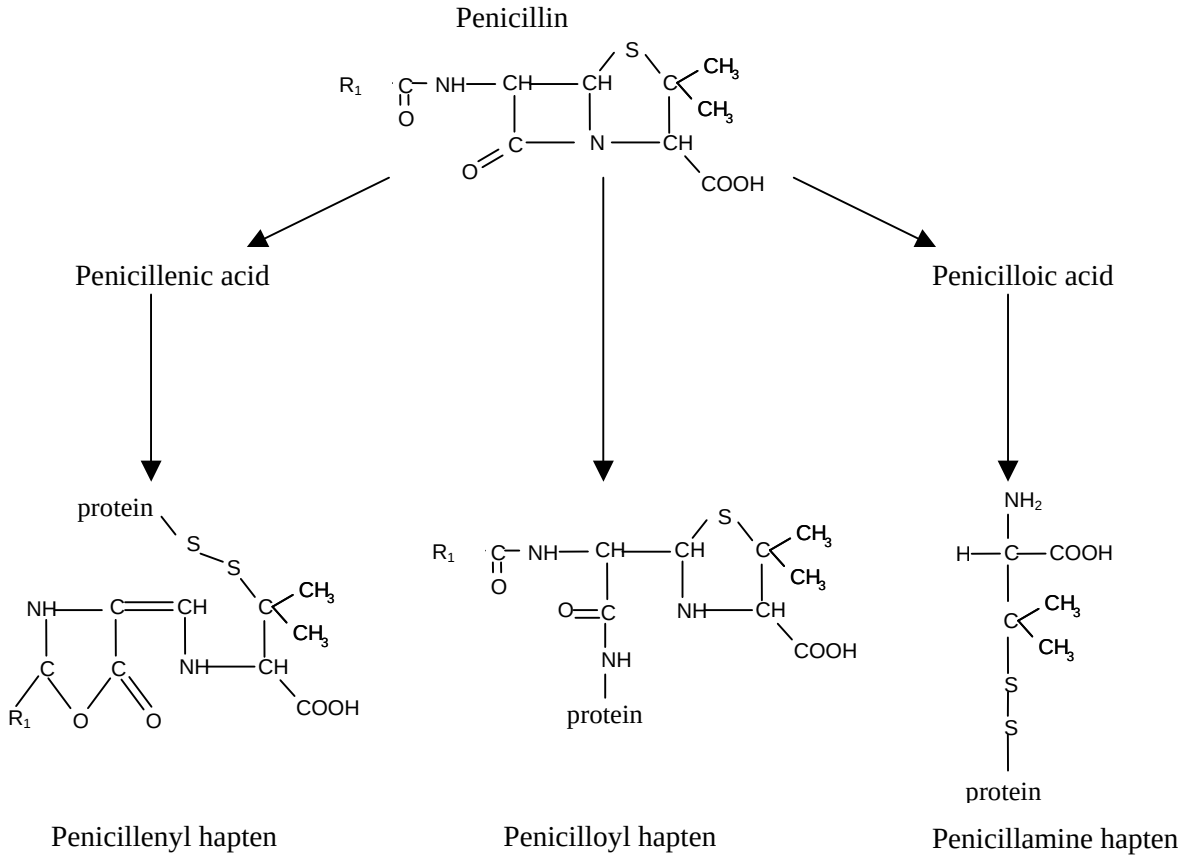


Figure 2.2: Haptens derived from penicillin.

2.4. Implication of penicillin residues

The use of penicillins in veterinary medicine can lead to penicillin residues in edible tissues and milk of food producing animals, to an increased bacterial resistance against these antibiotics, to the transfer of these resistant bacteria from animals to humans, and to adverse reactions in some individuals after consumption of food of animal origin with low levels of antibiotic (Boison, 1995; Anonymous, 1997b).

2.4.1. Bacterial resistance to penicillins

The use of antimicrobials leads to the selection of resistant bacteria. The development of resistance is influenced by drug concentration, long-term exposure, organism type,

antimicrobial type and host immune status. Low-level, long-term exposure to antimicrobials (feed additives) may have a greater selectivity than short-term, full-dose therapeutic use. It is suggested that levels of antimicrobials exceeding the MRL in food of animal origin represent low risks for the generation of resistance in humans. However, the presence of antimicrobials at concentrations above the MRL indicates inappropriate use of antimicrobials by the producer (Anonymous, 1997b).

Resistance to penicillins is mainly mediated by a large number of β-lactamases. Other resistance mechanisms include the acquisition of penicillin binding proteins (PBPs) with reduced affinity for β-lactams, mutations in the PBPs, and also reduced β-lactam uptake due to alterations in the outer membrane of Gram- bacteria or export by multidrug transporters (Schwarz and Chaslus-Dancla, 2001; Butaye et al, 2001).

2.4.2. Adverse reactions to penicillins

Despite their low toxicity, residues of penicillins in food can be harmful for the consumer (Milhaud and Person, 1981), by eliciting allergic responses. Penicillins are able to induce the four types of hypersensitivity: immediate hypersensitivity reactions including anaphylaxis (Type I), antibody-dependent complement-mediated cell lysis (Type II), immune complex reactions (Type III) and delayed hypersensitivity (Type IV). However, the Type I or IgE-mediated reactions are most significant. The immuno-allergic properties of the β-lactams are related to the reactive structure of the parent drug and some major metabolites. As hapten, they can covalently bind macromolecules. The major determinant is the penicilloyl conjugate formed between the carboxyl group of the open β-lactam ring and the amino group of the macromolecule (Dewdney et al, 1991).

It is considered unlikely that a person will develop allergic reactions after intake of penicillin contaminated food since allergic reactions are least likely to occur when the drug is administered orally (Dewdney et al, 1991). Moreover, it is assumed that no toxic or allergic reactions will occur if the MRL is not exceeded (Okerman, 1995). However, the risk in case of already sensitised individuals may not be underestimated (Dewdney et al, 1991). Indeed, penicillin is the most common cause of allergic drug reactions; it is estimated that penicillin allergy occurs in 3 to 10 % of the general population (Raynor, 1997).

2.4.3. Technological implications

Starter cultures (e.g. lactic acid bacteria) used in fermentation processes are sensitive to antibiotics. Several studies report the influence of penicillin antibiotics on the fermentation process of yoghurt, cheese and sausage preparation (Mourot and Loussouarn, 1981; Allison, 1985; Koenen-Dierick and Van Hoof, 1988; Grunwald and Petz, 2003). However, the MRL is lower than the levels required to inhibit the growth of starter culture (Bergstrom, 1996), so that in case of correct use such problems should not be expected.

2.4.4. Environmental risks of penicillins

Until now, only one study is published about β-lactam contamination of feed for animals giving rise to too high levels of residues in edible tissues and milk of the animals after intake of the contaminated feed (McEvoy, 2002). McEvoy (2002) described the occurrence of penicillin V in milk tank samples from two farms in the UK, while penicillin V has no MRL for milk and is only authorized as feed additive for pigs. It was concluded that the presence of the residues could only have been caused by feeding penicillin V contaminated feed to dairy cows.

2.5. Current state on detection methods

A lot of screening tests are available for the detection of penicillins. However, most of them were developed for the analysis of milk and are not suitable for the detection of penicillin in animal tissues. Moreover, microbiological inhibition tests, receptor tests and enzymatic assays only detect the microbiological active form of penicillins and not the degradation products. Penicillins are easily broken down (open beta-lactam ring), especially during sample storage and preparation (Boison, 1995). As a result, an underestimation of the real penicillin concentration in the carcass will occur. Another drawback of the microbiological inhibition tests and some of the receptor tests is that they do not discriminate between different antimicrobial families. These assays are thus suitable for pre-screening purposes.

Assays using antibodies specific for an epitope of the common penicillin structure, but different from the beta-lactam ring could circumvent these problems. Such antibodies should

allow the detection of both the native penicillin molecule and the degradation products. Several studies have been published about the development of penicillin-specific antibodies (de Haan et al., 1985; De Leuw et al., 1997; Dietrich et al., 1998; Usleber et al., 2000), but only some of them gave rise to the development of a group-specific penicillin assay, like the LacTek™ ELISA (Mitchell et al., 1999; not available anymore) and the Parallux™ assay (Huth et al., 2002).

Optical biosensors, such as BIAcore™ are becoming widely considered for food quality and safety control. The BIAcore™ technology provides fast, automated, reliable, robust and high capacity multi-residue analysis. Analysis of one sample is completed within minutes. At the moment, no test kits for penicillins are commercially available.

Among all kind of physico-chemical techniques, liquid chromatography coupled to spectrometry is very efficient for the detection of all penicillins at the MRL in animal tissues (Boison, 1995). This technique, like most of the physico-chemical methods, is also very expensive and laborious and can therefore only be applied for confirmation purposes.

Some of the currently used pre-screening (detection of an antimicrobial agent) and screening assays (group identification) are mentioned below. The confirmation methods will not be discussed in this review.

2.5.1. Microbiological inhibition assays

Penicillins are detected below the MRL in kidney and muscle tissues using the New Belgian Kidney Test (NBKT) and the European Four Plate Test or a variant (Okerman, 1995, Okerman, 1998b). These microbiological inhibition assays do not discriminate between different families of antimicrobial agents. However, repeating the assays in the presence of Penicillinase I allows to identify penicillins. Indeed, the enzyme will abolish the antimicrobial activity of a penicillin present in the suspected samples.

Other broad-spectrum screening tests based on the inhibition of bacterial growth are the Delvo®test SP and the Premi®test (DSM-Gist, Delft, Netherlands). Both assays contain spores of *Bacillus stearothermophilus* in agar together with a pH-indicator. The colour change

of the agar medium caused by the acid production is interpreted visually. In Belgium, the Delvo®test SP is used as confirmatory method for the detection of penicillins in milk (Reybroeck and Ooghe, 2003). No studies or data are available for the application of the Delvo®test on meat samples. The Premi®test is specially designed for meat and meat products. Penicillins are detected in spiked meat fluids from pig, chicken and cattle at levels ten times below the MRL (Arts et al, 2000). The drawback of the Premi®test is the visual interpretation of the colour development. However, the use of scanner measurements to determine the test end-point was recently reported, improving the assessment of the analytical data by eliminating the subjectivity of the previous visual approach (Stead et al., 2004). This assay seems very promising for pre-screening purposes.

2.5.2. Receptor assays

Three receptor assays are commercially available.

The Beta-STAR (UCB Bioproducts) uses a penicillin-specific receptor and an immuno-chromatographic support, and is designed for the detection of penicillins in milk. An extraction procedure was proposed for the analysis of porcine tissues, enabling the detection of penicillins at the MRL. The detection was however close to the limit of detection of the assay (Janosi et al., 2000).

The Charm II test is based on the competition between analyte in the test sample and radioactive labelled analyte for the binding on an analyte-specific receptor (Charm and Chi, 1988). The assay was initially developed for milk, but has since been modified to include tissues (Lynas et al, 2000). Penicillins, but also cephalosporins are detected below the MRL. The drawback of the method is the use of radioactive labelled analytes.

The Penzyme test is based on the inactivation of the enzyme carboxypeptidase by binding of beta-lactams. The assay was also initially developed for analysis of milk. Everest and coworkers (1993) described a modification of the method for the analyses of kidney tissues. Nevertheless, until now the application of the assay for screening purposes of animal tissues has not been reported.

2.5.3. Immunoassays

The Parallux™ assay is a solid-phase fluorescence immunoassay developed for the detection of antibiotics in milk (Huth et al., 2002). The test system is designed as cartridges, each with 4 channels (tests): one cartridge can be used to detect four different analytes in the same sample, or one or 2 analytes in 4 or 2 different samples, respectively. Using the β-lactam channel, ampicillin, amoxicillin, benzylpenicillin can be detected at 4 ppb, cloxacillin at 20 ppb and dicloxacillin at 50 ppb in milk. With the cloxacillin channel, cloxacillin and dicloxacillin can be detected at 8 and 10 ppb, respectively, in milk. Okerman and coworkers (2003) investigated the use of the Parallux™ for the analyses of several antibiotics in bovine and porcine kidneys. Using the penicillin cartridge, benzylpenicillin, amoxicillin and ampicillin were easily detected at the MRL. The assay was found to be suitable for the second screening step where positive samples are analysed to identify the group of antibiotics.

Chapter 3

Sulfonamides

Chapter 3: Sulfonamides

3.1. Introduction: use of sulfonamides in veterinary husbandry

The discovery of sulfonamides dates from 1932 when Gerhard Domagk and coworkers showed that mice infected with *Streptococcus pyrogenes* could be protected from peritonitis by the chemically synthesized sulfanilamide. Since then, sulfonamides are widely used in human and veterinary medicine for the treatment of bacterial infections. Unlike in human medicine, they are also applied as feed additive for growth promotion and prevention of bacterial infection in food-producing animals (Dupont and Steele, 1987; Long et al. 1990; McEvoy, 2002). Many parental, intramammary and oral preparations are authorized for the treatment of a variety of conditions in domestic and food producing animals in the EU (McEvoy, 2002). In Europe, Canada and the United States, the MRL for the total amount of sulfonamides in edible tissues and milk is 100 µg/kg. In Japan, this MRL is 20 µg/kg (Anonymous, 1990; Haasnoot et al, 2000a). There is no MRL for eggs, and there are no products authorized for laying-hens in the EU. For medicated feedingstuff, the inclusion rates for sulfonamides range from 73 to 812 mg/kg for pig and poultry and from 1250 to 5000 mg/kg in fish feeds (Anonymous, 1996b).

3.2. Physicochemical and biological properties

The common structure of sulfonamides, sulfanilamide, is composed of a benzene ring bearing the sulfonamide group ($-SO_2NH_2-$) at position 1 and an aromatic amino group ($-NH_2$) at position 4 (Figure 3.1). Derivatives of sulfanilamide are formed by substitution of the hydrogen of the sulfonamide group or the aromatic amino group. Sulfonamides with antibacterial activity have no substitution at the aromatic amino group. The sulfonamides are amphoteric and have different pKa values (SO_2-NH; sulfanilamide: 10.43; sulfamethoxine: 7.4; sulfadiazine: 6.4; sulfachloropyridazine: 5.1). Most sulfonamides are uncharged compounds between pH 5.0 and 5.2 (Guggisberg et al, 1992). Sulfonamides are usually very soluble in organic solvents like methanol, aceton and alcohol while poorly soluble in aqueous solutions, diethylether and chloroform. The solubility increases with increasing pH and temperature (Budavari, 1988). Sulfonamides are rather stable molecules, only a few being sensitive to UV radiation (Guggisberg et al., 1992).

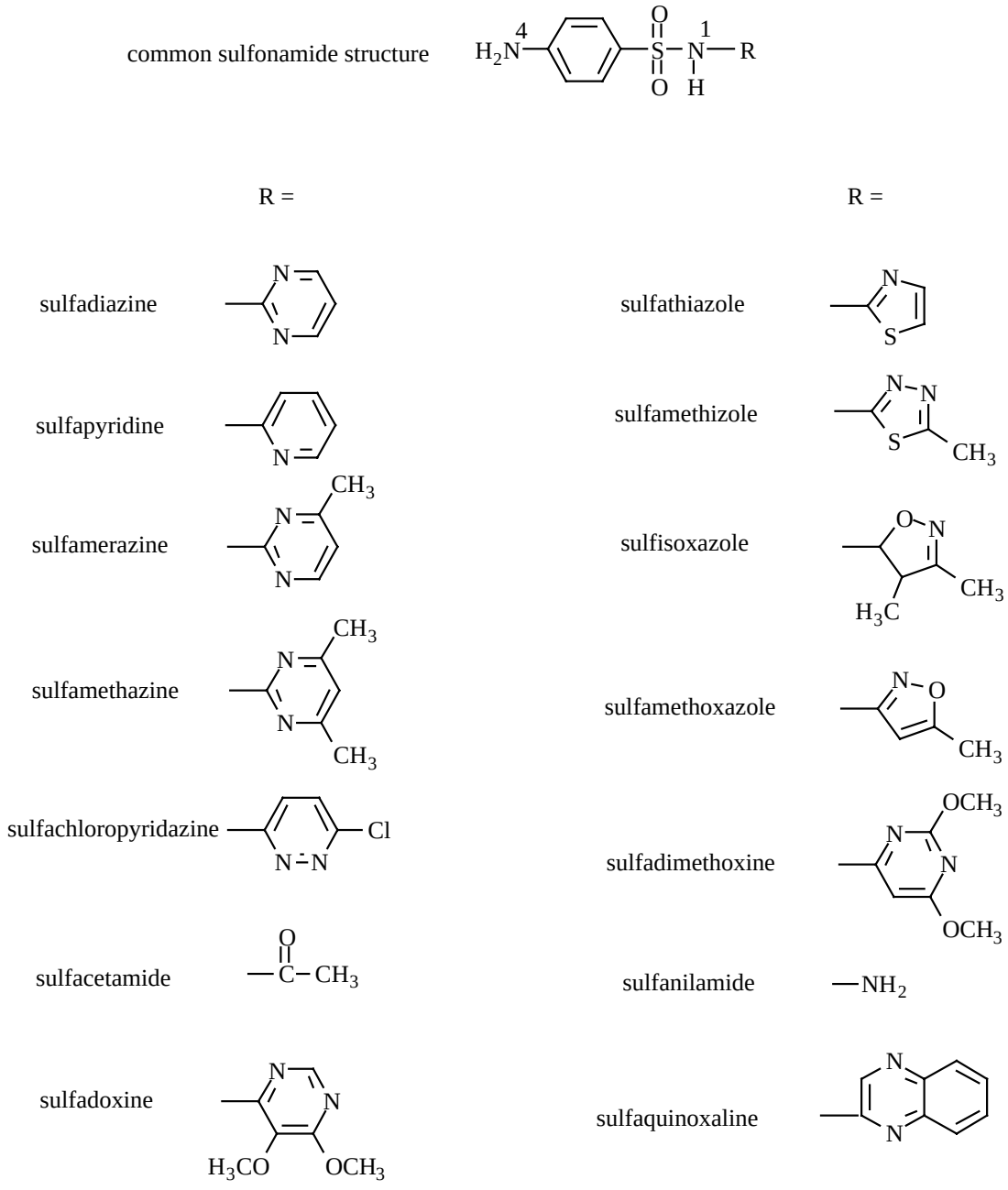


Figure 3.1: Structure of sulfonamides

The antibacterial activity of sulfonamides is due to the inhibition of the folic acid biosynthesis in micro-organisms. Folic acid is, as it is for humans, essential for the synthesis of nucleic acids. Mammalian cells utilize dietary folates. Prokaryotes, however, have to synthesize the molecule by a cascade of reactions, including the formation of dihydropteroic acid catalysed by dihydropteroate synthase (DHPS). Sulfonamides are structural analogues of para-aminobenzoic acid (PABA). The target of the sulfonamides is the enzyme dihydropteroate synthase (DHPS), that catalyses the condensation of PABA and 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid.

3.3. Metabolization of sulfonamides

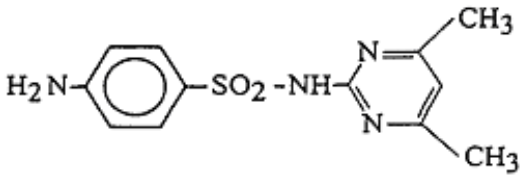
Drugs like sulfonamides are eliminated from the body as unchanged compounds or after extensive metabolization. Excretion of the parent drug and the metabolites may occur via urine, bile, faeces or milk depending on their physicochemical properties.In general, the resulting metabolites possess a higher polarity and a better water solubility than the parent drug, favouring the renal excretion (Rehm et al., 1986). The metabolization and pharmacokinetic aspects of sulfonamides depend on various parameters like dose, route of administration and molecular structure of the sulfonamide, but also on the age and species (Nouws et al., 1989).

The different metabolic pathways for sulfonamides are acetylation/deacetylation, hydroxylation, glucuronidation, sulfatation and deamination (Rehm et al., 1986).

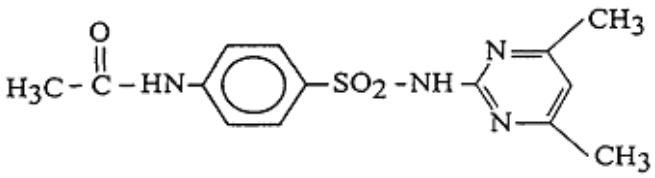
Acetylation is the major metabolic pathway for most sulfonamides and occurs in all tissues. There is an equilibrium between acetylation/deacetylation. The most occurring acetyl metabolite is the N^4-acetyl derivative. In humans, acetylation predominates, while deacetylation in pigs, horses and cattle (Nouws et al., 1988). Glucuronidation was observed in milk and urine, but no data are available about occurrence of this pathway in tissues (Rehm et al, 1986). However, glucuronide metabolites are formed post mortem in tissues during storage (Alfredsson and Ohlsson, 1998). Metabolites formed by hydroxylation can be harmful and may cause allergic reactions (Meekins et al., 1994; Knowles et al., 2001). Deamination, in contrast to the other pathways, decreases the polarity en thus the excretion of sulfonamides. This pathway is preferred in case of low acetylation rate and is therefore not important in humans. Deamination however, seemed to be important in poultry. Desamino-metabolites are

probably formed by bacteria in the gut after oral administration of sulfonamides (Crabbe, 2002). Sulfatation occurs in very low amounts (Rehm et al, 1986). The chemical structure of sulfamethazine and its main metabolites are shown in figure 3.2.

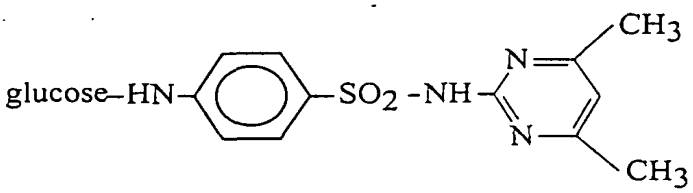
sulfamethazine (SMT)



N_4-acetyl-SMT



N_4-glucoside of SMT



desamino-SMT

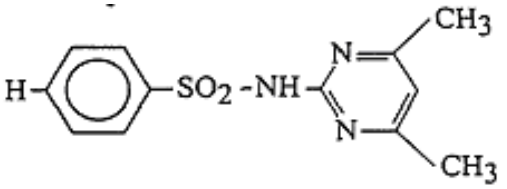


Figure 3.2.: Structure of sulfamethazine and its main metabolites

3.4. Implication of sulfonamides residues

3.4.1. Bacterial resistance to sulfonamides

A special problem is the effect of sulfonamides on the human intestinal microflora. It is well known that an antibiotic treatment can affect the intestinal microflora of the patient. Most likely, the same effect will be obtained after routinely intake of food containing antibiotics. The intestinal human microflora is composed of anaerobe Gram+ and Gram– bacteria. They are responsible for the "barrier-effect" or the inhibition of the colonization by obligate aerobe and resistant aerobe micro-organisms. Oral administration of antibiotics can disturb the anaerobe microflora and affect the barrier-effect, and consequently allow the pathogens to develop.

Another mechanism influencing the intestinal microflora is the selection of antibiotic resistance. It is assumed that the continuous intake of antibiotics over a longer period can promote the occurrence and spreading of resistant micro-organisms. The minimal inhibitory concentration (MIC) is the concentration required for the inhibition of normal sensitive bacteria. The MIC of sulfonamides is 25 to 100 µg/ml. The selection can only occur if the concentration of antimicrobial that reaches the intestinal flora is as high as the MIC for this antimicrobial drug in relation to the normal sensitive intestinal microflora. In this case, the growth of sensitive microorganisms is inhibited and consequently the development of resistant bacteria is promoted. Since the MRL for sulfonamides is 100 ng/ml, it is assumed that a resistance selection due to sulfonamides present in food can only occur when the concentration of sulfonamides exceeds the MRL (Haesebrouck and Devriese, 1994).

However, the most important risk of the misuse of antibiotics is the transfer and spreading of resistant microorganisms to the environment and to humans (Guillot, 1989; Franco et al, 1990). Several studies have demonstrated the correlation between the use of antimicrobials in food producing animals and the occurrence of resistant bacteria in the intestinal microflora as well as among pathogens. The consumer can be infected with these resistant bacteria. Moreover, the resistance genes can be transferred between bacteria, and even between different bacterial families (van den Bogaard and Stobberingh, 1999). Identical resistant genes have been found in different bacterial species (Bergstrom, 1996). For sulfonamides, two resistance genes, *sul1* and *sul2*, were characterized in Gram- bacteria. They

produce distinct DHPS molecules (57 % amino acid identity), which show pronounced insensitivity to sulfonamide inhibition at a normal substrate saturation (Skold, 2000; Schwarz and Chaslus-Dancla, 2001).

3.4.2. Adverse reactions to sulfonamides

Due to the diversity in molecular structure of different sulfonamides, the allergenicity of sulfonamides varies considerably (Tilles, 2001). Sulfonamides cause allergic reactions, which range from fever and skin rash to urinary and hepatopoietic disorders. Other organs, such as the heart, kidney and lung, can also be involved (Ahmad et al, 2002). Studies about sulfonamide allergies always discuss the incidence of allergic reactions after intake of the chemotherapeutical as medicine (Knowles et al, 2001; Trepanier, 1999). No data are available about the occurrence of allergy after consuming food containing sulfonamides. It is assumed that no toxic or allergic reactions will occur if the MRL is not exceeded (Okerman, 1995). However, it cannot be excluded that sulfonamides, as penicillins, could trigger an allergic reaction in already sensitised individuals. While sulfonamide hypersensitivity is relatively rare in the general human population, it is very important for HIV patients. Fifty to 80 % of patients with HIV treated with sulfonamides develop adverse reactions (Trepanier, 1999).

Sulfonamides may be responsible for the induction of acute toxicity (Poirier et al., 1999). Sulfamethazine is a suspected carcinogen (Sternesjo et al, 1995). Less data are available about the chronic toxicity of sulfonamides.

3.4.3. Environmental risks of sulfonamides

Some of the antimicrobial drugs used in veterinary medicine will be excreted through faeces and urine and finally reach the soil and water. Sulfonamides are very stable molecules (Guggisberg et al., 1992). Bioaccumulation of sulfadimethoxine has been reported in crops after growth on contaminated soil (Bergstrom, 1996).

Several studies have demonstrated the presence of sulfamethazine in edible tissues of pigs that received non-medicinated feed (Bevill, 1984; Mc Evoy, 2000; McEvoy, 2002). The contamination was due to the carry-over of sulfamethazine between medicinated and non-medicinated feeds during the production process. The effect of such a carry-over on

exceeding the MRL will depend on the molecule as well as the species affected. Indeed, several studies reported that equivalent low concentrations of sulfathiazole or sulfadiazine in feeds did not lead to violative levels in edible porcine tissues (Bevill, 1984; McEvoy, 2002). While not allowed for use in laying hens, sulfadiazine has been detected in eggs (McEvoy, 2002). Although the reason for the violation was not stated, it is likely that feed contamination was the cause.

3.4.4. Implication of sulfonamide metabolites

The MRL for sulfonamides is determined for the parent drug molecule and not for the metabolites. The metabolites are not a risk for the human health when the concentration of sulfonamides in food products does not exceed the MRL, because the retention time of metabolites in the human body is too short (Nouws et al., 1985). Furthermore, metabolites should not be detected in order to avoid technical implications during production processes and development of resistant pathogenic bacteria since sulfonamide metabolites are microbiological inactive after acetylation, glucuronidation, sulfatation and deamination. Only hydroxylation metabolites remain active. However, sulfonamides undergo different metabolizations and therefore it is improbable that the remaining metabolite is still active (Rehm et al., 1986).

For drug monitoring purposes, the formation of glucuronide metabolites during storage of samples should be considered. Since incurred samples cannot always be tested immediately, the formation of glucuronide metabolites will cause false-negative results. The native sulfonamide molecule however can be formed by heating or under acidic conditions (Alfredsson and Ohlsson, 1998; Smit et al, 1999). Glucuronide metabolites are usually not detected with the current methods. It is therefore advisable to incorporate a de-glucuronidation procedure during the sample preparation.

3.5. Current state on detection methods

At the moment, only a few tests are suitable for the detection of sulfonamides at the MRL in meat samples. The problem of sulfonamides is that they are poorly water-soluble. Usually, simple extraction or even no sample preparation is applied for the analyses in screening assays. Sulfonamides remain thus in the matrix. Most microbiological assays are not sensitive enough for sulfonamides to allow an efficient extraction. Several studies

describe the development of sulfonamide-specific antibodies. These studies are discussed in Chapter 8. None of them gave rise to a commercially available group-specific sulfonamide assay.

As for penicillins, liquid chromatography coupled to spectrometry is very efficient for the detection of all sulfonamides at the MRL in animal tissues (Van Eeckhout et al., 2000; Ito et al., 2000). This technique, like most of the physico-chemical methods, is very expensive and laborious and can therefore only be applied for confirmation purposes.

Some of the currently used or new pre-screening (detection of an antimicrobial agent) and screening assays (group identification) are mentioned below. Most assays have already been treated for penicillins. The confirmation methods will not be discussed in this review.

3.5.1. Microbiological inhibition assays

For pre-screening purpose, the broad-spectrum New Belgian Kidney Test (NBKT) is recommended by the Belgian legislation (Anonymous, 1995), but the assay is not sensitive enough (Okerman, 1995). The European four plate test or variants can be applied for group identification, but these assays are also not sensitive enough (Okerman et al., 1998a; Okerman et al., 1998b). The use of the Premi® test (DSM-Gist, Delft, Netherlands), also a broad-spectrum microbiological inhibition assay, could offer an alternative (Arts et al., 2000; Reybroeck, 2000; Stead et al., 2004).

3.5.2. Receptor assays

The receptor assay Charm II is able to detect sulfonamides below the MRL (Charm and Chi, 1988; Nolan et al., 2000). The drawback of this method is the use of radioactive labelled agents.

3.5.3. Immunoassays

A lot of ELISA’s are commercially available, each of them highly specific for one or two sulfonamides. However, it would be more efficient to have one immunoassay able to detect all sulfonamides instead of several immunoassays, each specific for an individual sulfonamide.

A cartridge for the detection of three sulfonamides is available in the Parallux™ assay, enabling the detection of sulfamethazine, sulfadimethoxine and sulfathiazole in one sample.

Recently, a test kit for the analyses of sulfonamides (Qflex® Kit Sulfonamides) using the optical biosensor Biacore®Q has become commercially available. The assay uses a sulfonamide-specific monoclonal antibody and is validated for the analysis of pork muscle tissues (McGrath et al., 2004).

3.5.4. Thin Layer Chromatography

Due to the lack of a sensitive and group-specific screening assay for sulfonamides, thin layer chromatography (TLC) is currently used. Gugissberg and coworkers (1992) published a review of the different TLC methods for the detection of sulfonamides in meat. Different techniques for the visualisation of sulfonamides on TLC-plates are available. The advantage of TLC compared to other chromatographic methods is the possibility of analysing several samples simultaneously rather than serially. Newer TLC-materials allow shorter run times, resulting in significant timesavings.

The commercial Sulfa-On-Site test is a TLC-based method for the detection of sulfamethazine in porcine urine. The test uses a correlation between sulfamethazine levels in urine and tissues, to estimate the concentration of sulfamethazine in tissues. Although claimed to be simple and accurate, the method was found not suitable for on-farm monitoring (Shearan and O'Keeffe, 1994)

Part II

Aims of the study

Aims of the study and framework

The general aim of this study was to develop group-specific screening assays using antibodies. Hereto antibodies had to be produced that were specific for the common structure of a group of antimicrobials. In the present thesis, two groups of antimicrobials were selected namely penicillins and sulfonamides. Penicillins were chosen since it is well known that people or animals which have been shown to be allergic for a penicillin are also allergic for the other penicillins. This indicates that the common structure is immunogenic and penicillins would be ideal to test the hypothesis that group-specific screening assays could be developed. Sulfonamides were chosen since it is an important group for which no easy to perform screening assays are available.

Group-specific antibodies should allow the development of an immunochemical screening assay, like an enzyme-linked immunosorbent assay (ELISA) or a biosensor assay. In combination with an appropriate extraction procedure, the ELISA's or biosensor should then be applied for the detection of penicillins, respectively sulfonamides in meat samples.

So following questions were posed:

1. Is it possible to develop antibodies recognizing a group of antimicrobials?
2. Can a screening assay be developed using group-specific antibodies?
3. Is it possible to develop one extraction procedure for a group of antimicrobials?

Different strategies were applied for the development of group-specific monoclonal antibodies. The search for penicillin-specific antibodies is described in chapter 4, and for the sulfonamide-specific antibodies in chapter 8.

Using the most successful immunogens for monoclonal antibody production, polyclonals were induced. In chapter 5 and 9, the production of polyclonal antibodies specific for penicillins and sulfonamides, respectively, are described.

Chapter 6 analyses and compares the penicillin-specific antibodies in ELISA and in the Biacore optical biosensor. A comparison between monoclonals and polyclonals is made.

The application of an ELISA for the analysis of meat samples is examined in chapter 7 for penicillins and in chapter 9 for sulfonamides.

Finally, in the general discussion, the results obtained for penicillins and sulfonamides are compared.

Part III
Immunochemical detection of penicillins

Chapter 4

Generation of class-selective monoclonal antibodies against the penicillin group

Based on: P. Cliquet, E. Cox, C. Van Dorpe, E. Schacht and B. M. Goddeeris. Generation of class-selective monoclonal antibodies against the penicillin group. J. Agric. Food Chem., 2001, 49, 3349-3355.

Abstract

In order to develop a penicillin-specific ELISA, different attempts were made to obtain monoclonal antibodies specific for the common structure of penicillins. Ampicillin was coupled to different carrier-proteins (bovine serum albumin, chicken ovalbumin and thyroglobulin) to render it immunogenic. Different coupling methods were compared: two methods using a cross-linker (glutaraldehyde or a succinimide ester), one carbodiimide-mediated coupling method and one method without any cross-linker or mediator molecule (physiological binding). Mice were immunized with the conjugates intraperitoneally or in the footpad. A screening-ELISA was developed to detect anti-ampicillin antibodies in sera. Specificity and affinity of the antibodies were demonstrated by inhibiting their binding with a 10 mM solution of ampicillin. No difference could be observed using electrofusion or PEG-mediated fusion. For the production of the monoclonals, an intravenous final boost gave antibodies with better specificity and affinity than an intraperitoneal final booster injection. At least one anti-ampicillin monoclonal antibody (19C9) cross-reacts with penicillin G, oxacillin, dicloxacillin and carbenicillin, and not with sulfanilamide, chloramphenicol, neomycin and streptomycin, and is therefore interestingly to be considered for developing a penicillin-specific ELISA.

Keywords: β-lactam antibiotics - immunogenicity – enzyme-linked immunosorbent assay - monoclonal antibodies

Introduction

Penicillins are widely used in veterinary medicine. As a result, food derived from animals treated with antibiotics, may be contaminated with these drugs. To protect consumers from risks related to drug residues, maximum residue levels (MRL) are determined by law (Anonymous, 1990). To analyse presence or absence of penicillin residues in meat and milk products, microbial inhibition tests and receptor assays are most commonly used (Allison, 1985; Charm and Chi, 1988; Kavanagh, 1989). These methods either are not specific enough or do not allow the detection of the whole group of penicillins. Physico-chemical methods, like high-performance liquid chromatography (HPLC), need time-consuming sample preparation and only one sample can be handled at a time. An alternative for the restrictions

inherent to these techniques is the detection of residues by enzyme-linked immunosorbent assays (ELISAs) (Paraf and Peltré, 1991). ELISAs are quick, specific, sensitive and have the additional advantage of analysing several samples simultaneously.

The aim of the present study was to develop an ELISA specific for the group of penicillin antibiotics. As β-lactam antibiotics share a 6-aminopenicillanic acid structure (a β-lactam ring structure coupled to a thiazolidin ring, figure 2.1, chapter 2) and differ in their acyl-side chain, the strategy followed was to induce and select monoclonal antibodies directed against the common 6-aminopenicillanic acid core. Using ampicillin-protein conjugates, studies on the antigenicity of penicillins have indicated three important epitopes: the acyl-side chain, the common thiazolidin ring and newly formed structures that arise by coupling penicillins to a carrier-protein (de Haan et al., 1985; Mayorga et al., 1995). In the present study, the immunogenicity in mice of different ampicillin-conjugates was compared and the characteristics of the produced ampicillin-specific monoclonal antibodies were determined.

Material and methods

Reagents and chemicals

Benzylpenicillin, amoxicillin, 6-aminopenicillanic acid, bovine serum albumin (bsa), thyroglobulin, ovalbumin (ova), glutaraldehyde, hydroxylamine, tetrahydrofuran, 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC), *s*-acetylmercaptosuccinic anhydride (SAMSA), 3-maleimidobenzoic-*N*-hydroxisuccinimide ester (MBS), Ellman's reagent (= 5,5'-dithio-bis(2-nitrobenzoic acid), citraconic anhydride, Bicinchoninic acid (BCA), Cupper(II)sulphate pentahydrate ($CuSO_4.5H_2O$) and OPI supplement media were purchased from Sigma-Aldrich (Bornem, Belgium). Oxacillin, dicloxacillin, and cloxacillin were obtained from ICN Biochemicals (Asse-Relegem, Belgium). Ampicillin, polyethylene glycol 1500 (PEG), ABTS (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) tablet® and ABTS buffer® were obtained from Roche Diagnostics (Brussels, Belgium). The ABTS substrate solution was prepared by dissolving 1 ABTS tablet® (5 mg) in 50 ml ABTS buffer®. Complete Freund's adjuvant (CFA) and incomplete adjuvant (IFA) were provided by Difco Laboratories, Biotrading (Bierbeek, Belgium). Tween 20® (polyoxyethylene sorbitan monolaurate) was purchased from Merck-Belgolabo (Overijse, Belgium). Rabbit anti-mouse immunoglobulins conjugated to peroxidase (α-mHRP prosan, code n°P0260) were obtained from DAKO Diagnostica, Prosan (Ghent,

Belgium). Dialysis tube VIKING (12 000 –14 000 MW cut off) was provided by ROTH, Fiers (Kuurne, Belgium). ELISA microtiter plates (maxisorp) were obtained from NUNC, Life technologies (Merelbeke, Belgium). Tissue culture plates were from Greiner (Wemmel, Belgium). Dulbecco modified Eagle's medium (DMEM), glutamine, gentamycin, sodium pyruvate, foetal calf serum (FCS) and hypoxanthine, aminopterin and thymidine supplement (HAT) were purchased from GibcoBRL, Life technologies (Merelbeke, Belgium). All other chemicals were of reagent grade or better. HAT-selection medium consisted of DMEM containing 20% FCS, 1% glutamine, 0,1% gentamycin, 1% sodium pyruvate, 1% OPI supplement media and 2% HAT. Phosphate buffered saline (PBS) solution (0.15 M pH 7.4) was prepared by dissolving 0.8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml distilled H_2O. The pH was adjusted to 7.4 with addition of NaOH or HCl. Finally the solution was made up to 1 L with distilled H_2O.

Carbodiimide mediated penicillin-carrier conjugation (Van Regenmortel et al., 1988; figure 4.1)

In order to protect the amino groups of the carrier protein for reacting with carbodiimide, the amino groups were blocked with citraconic anhydride. The carrier protein and citraconic anhydride were dissolved in 4 ml distilled H_2O in a molar ratio of peptide amino group:citraconic anhydride of 1:10. The pH was then continuously adjusted to 8.5-9 with 1 M NaOH until the pH remained constant. Subsequently, 100 µl citraconic anhydride was added. If the pH did not change, the reaction was gone to completion. Otherwise, the pH was again adjusted wit NaOH until it remained stable and the addition of citraconic anhydride was repeated. Next, the mixture was incubates during 15 min at RT under slow stirring. Subsequently, 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC) dissolved in 200 µl distilled H_2O, was added in a molar ratio of protein/MEDC of 1:10. After 5 min incubation at RT, ampicillin was added in a molar ratio of protein/amp of 1:100 for coupling to albumins, and in a molar ratio of 1:1000 for coupling to thyroglobulin. The mixture was then incubated for 2 hrs at RT. Deprotection of the amino groups of the carrier protein was done by dialysis (12 000-14 000 cut off) against 1 L of 5% acetic acid for 3 hrs at 4 °C. Finally, the product was dialyzed (12 000-14 000 cut off) for 24 hrs at 4 °C against 3 changes of 1 L PBS. Aliquots of the conjugates carbo amp-bsa, carbo amp-ova and carbo amp-thyro were stored at –20 °C.

The reaction scheme of the carbodiimide mediated conjugation of ampicillin is given in figure 4.1. During the reaction ampicillin polymers can be formed. These conjugates are probably removed by dialysis.

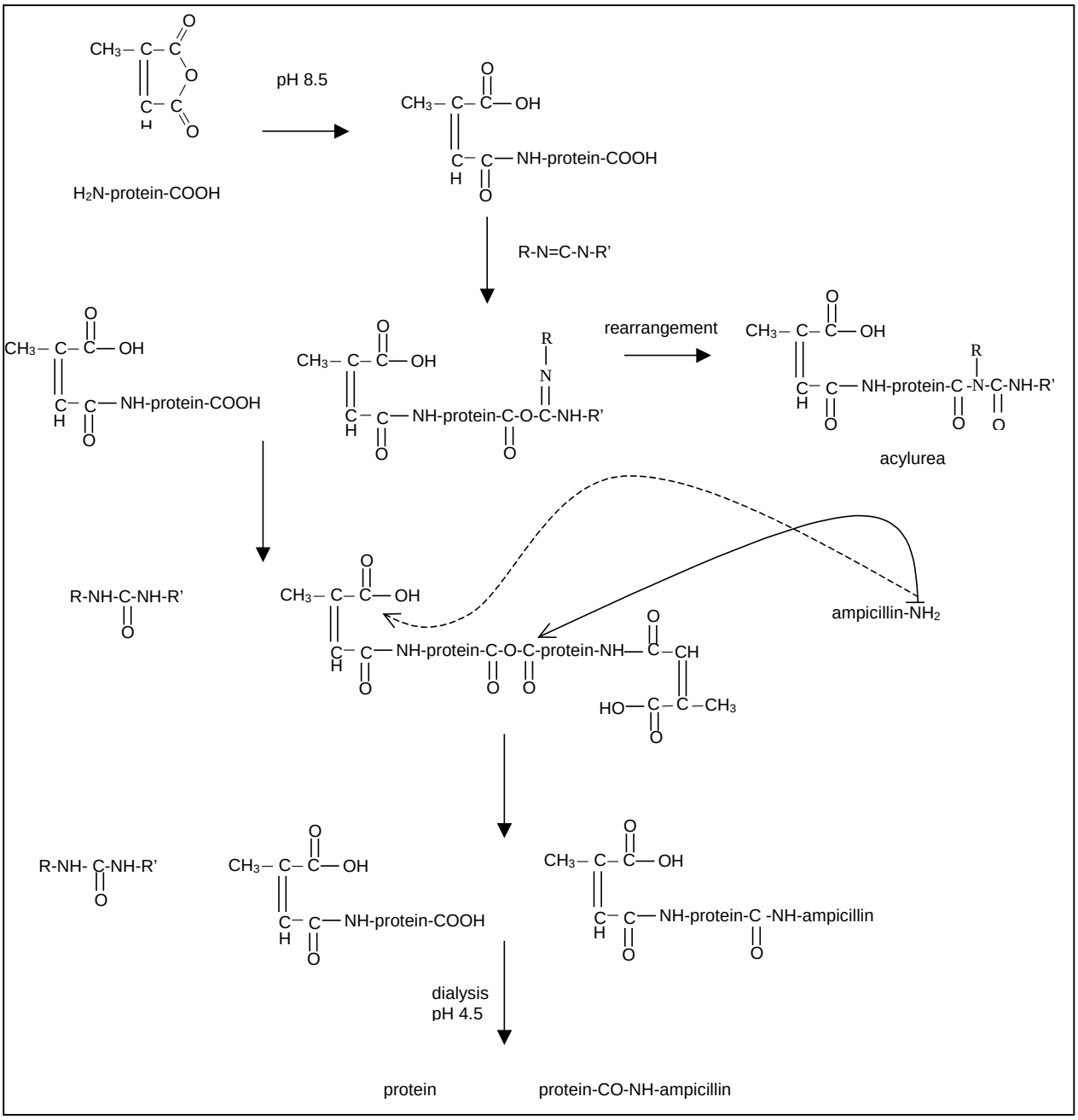


Figure 4.1: carbodiimide mediated conjugation method.

Preparation of penicillin-succinimide ester-carrier conjugate (van de Water, 1990; Kitagawa et al., 1988; figure 4.2)

a) Introduction of sulfhydryl groups on the carrier-protein (acetylthio-carrier-protein): 4.08 mmol *s*-acetylmercaptosuccinic anhydride (SAMSA) was added slowly to 0.077 mmol carrier-protein dissolved in 15 ml 0.1 M potassium phosphate buffer pH 7.3. Doing this, the pH was maintained between 7 and 7.5. Once all SAMSA was added, the pH was lowered to pH 6 by adding 1 N HCl. The solution was dialyzed during 1 week against distilled H_2O, whereafter the conjugate was lyophilized.

b) Removal of the acetyl group of acetylthio-carrier-protein: 10 µl deoxygenated 0.1 M hydroxylamine was added to 20 mg lyophilized acetylthio-carrier-protein dissolved in 500 µl deoxygenated 0.1 M phosphate buffer pH 7.3. The solution was then mixed under N_2 until no further increase in number of sulfhydryl groups could be observed. The number of sulfhydryl groups was determined using the Ellman standard method (Ellman, 1959). 20 µl Ellman's reagent (5,5'-dithio-bis(2-benzoic acid)) was added together with 20 µl sample to 1 ml sodium phosphate buffer 0.05 M pH 8 and incubated for 15 min at room temperature. The reaction between Ellman's reagent and free sulfhydryl groups results in a yellow colour that is measured with a spectrophotometer at 412 nm.

c) 3-maleimidobenzoic-*N*-hydroxisuccinimide ester (0.015 mmol MBS) dissolved in 0.5 ml tetrahydrofuran was added to 0.015 mmol ampicillin dissolved in 1 ml 0.05 M sodium phosphate buffer pH 7. The mixture was then incubated during 1 hr while gently stirring. Subsequently, tetrahydrofuran was removed by mixing the solution under N_2. The excess of MBS was removed by extraction with 3 times 5 ml methyleenchloride/ether (1:2; v/v). The aqueous phase contained the MBS coupled ampicillin (ampMBS) and was used in next step.

d) The thio-carrier-protein solution was added to the ampMBS solution and incubated for 2 hrs at 25 °C. The mixture was then dialyzed (12 000-14 000 cut off) against PBS during 3 days. Aliquots of the conjugates amp-MBS-ova and amp-MBS-bsa were stored at –20 °C.

The coupling efficiency was established by determining the number of sulfhydryl groups left after coupling (Ellman, 1959) and subtracting the amount from the number determined in step b. As only one ampicillin molecule can be coupled to one sulfhydryl group, the amount of bound ampicillin molecules equals the amount of reacted sulfhydryl groups: number of ampicillin molecules = $[(Ev-En)/Em]*f *N_A$

Ev = absorbance at 412 nm of the thio-carrier solution after reaction with Ellman's reagent

En = absorbance at 412 nm of the final product after reaction with Ellman's reagent

Em = molar extinction coefficient for the Ellman's reagent at 412 nm (13 600)

N_A = number of Avogadro

f = dilution factor

The amount of carrier-protein molecules was determined by measuring the protein concentration of the thio-carrier-protein solution via the absorbance at 280 nm before adding amp-MBS. The coupling efficiency is expressed as the number of ampicillin molecules bound to one carrier molecule in the final product (efficiency = number of ampicillin molecules / carrier molecule).

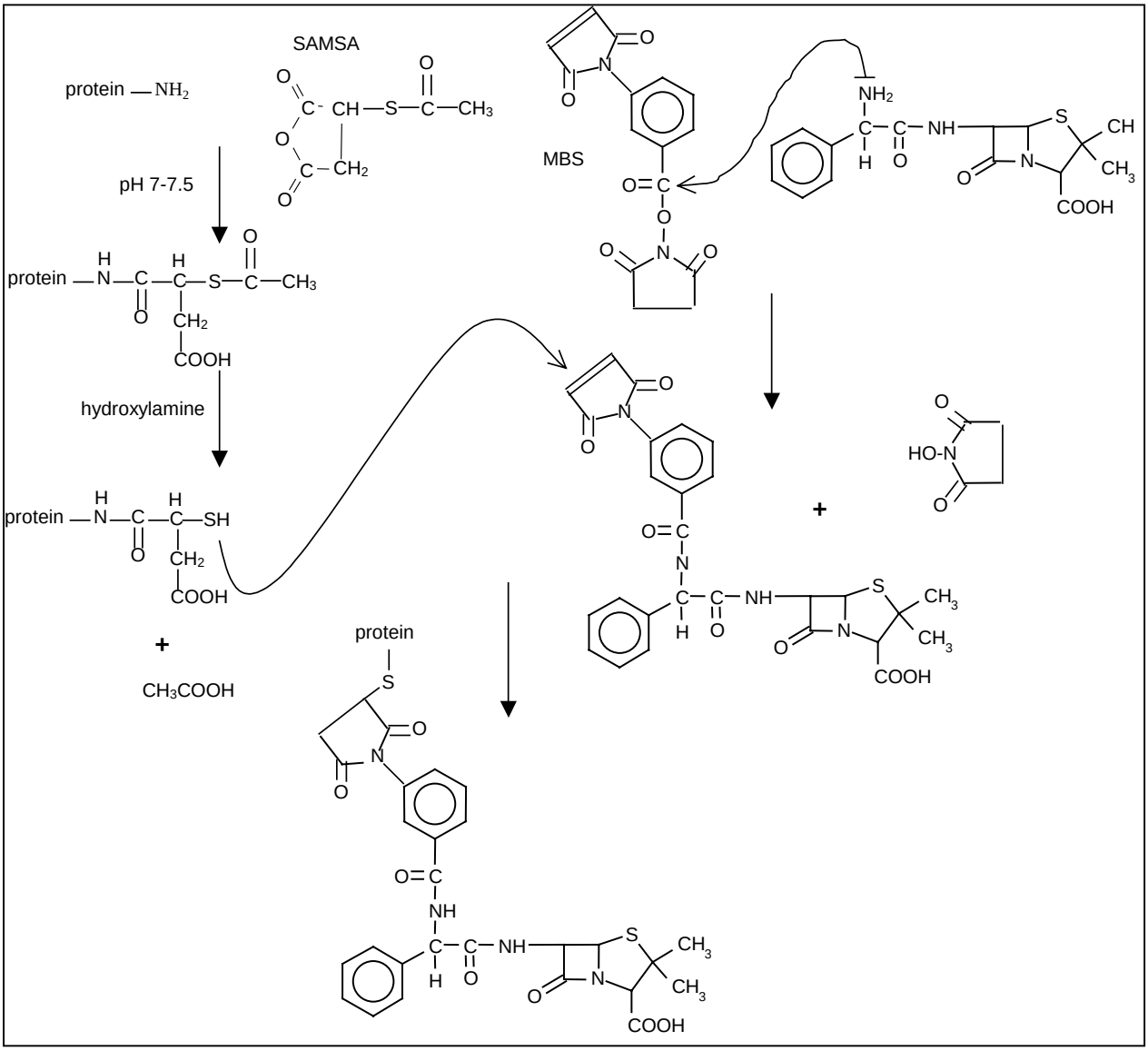


Figure 4.2: Conjugation of ampicillin to proteins using a succinimide ester coupling method

Preparation of penicillin-glutaraldehyde-carrier conjugate (Märtlbauer, 1993; figure 4.3)

Ampicillin (0.4 mmol) dissolved in 8 ml dimethylformamide was added to 0.003 mmol carrier-protein dissolved in 16 ml phosphate buffered saline 0.15 M pH 7.4 (PBS). Subsequently, 0.15 ml of glutaraldehyde (25%) was added drop wise to the solution. After 3 hrs gently stirring at RT, the reaction mixture was dialyzed (12 000-14 000 cut off) against PBS during 3 days. Aliquots of the conjugates amp-glut-bsa and amp-glut-ova were stored at –20 °C.

The reaction scheme of the glutaraldehyde reaction is given in figure 4.3. The coupling reaction is performed at neutral pH. Glutaraldehyde is then transformed in α,β-unsaturated aldehyde polymers that appear to form labile intermediate Schiff base with amino groups of the protein and ampicillin. The resonance interaction of the Schiff base with the double bond is believed to lead to a stable product. Another possible stabilization mechanism is a Michael-type addition that may occur at various sites when the local amine concentration is particularly high (van Regenmortel, 1988). The reaction can also be stabilized by addition of sodium borohydride.

In order to define the coupling efficiency the amount of carrier molecules had first to be determined by measuring the protein concentration of the final product via its absorbance at 280 nm. (penicillin shows no absorbance at 280 nm.). In a second step, the amount of bound penicillin in the final product was determined using the BCA method (Schmidt et al., 1985): 1 ml BCA-reagent (= 50 parts BCA + 1 part $CuSO_4.5H_2O$) is added to 50 µl of the sample whereafter the absorbance is measured at 562 nm after 30 min incubation at 37 °C. Because the carrier-protein in the final product also reacts with BCA reagent, a sample containing only the carrier-protein at a concentration equal to the one that was determined in step 1, was also used to react with BCA-reagent. The absorbance of penicillin in the final product equals the absorbance of the final product minus the absorbance of the sample with the carrier molecule at a concentration equal to the concentration determined in step 1. A calibration curve for ampicillin was established plotting the concentration of a standard dilution of ampicillin against the absorbance at 562 nm obtained for these standard samples after reaction with BCA-reagent. Extrapolation of the absorbance of penicillin in the final product lead to the concentration of penicillin in the product, and consequently to the amount of ampicillin molecules.

The efficiency of the reaction is expressed as the number of ampicillin molecules bound to one carrier molecule in the final product.

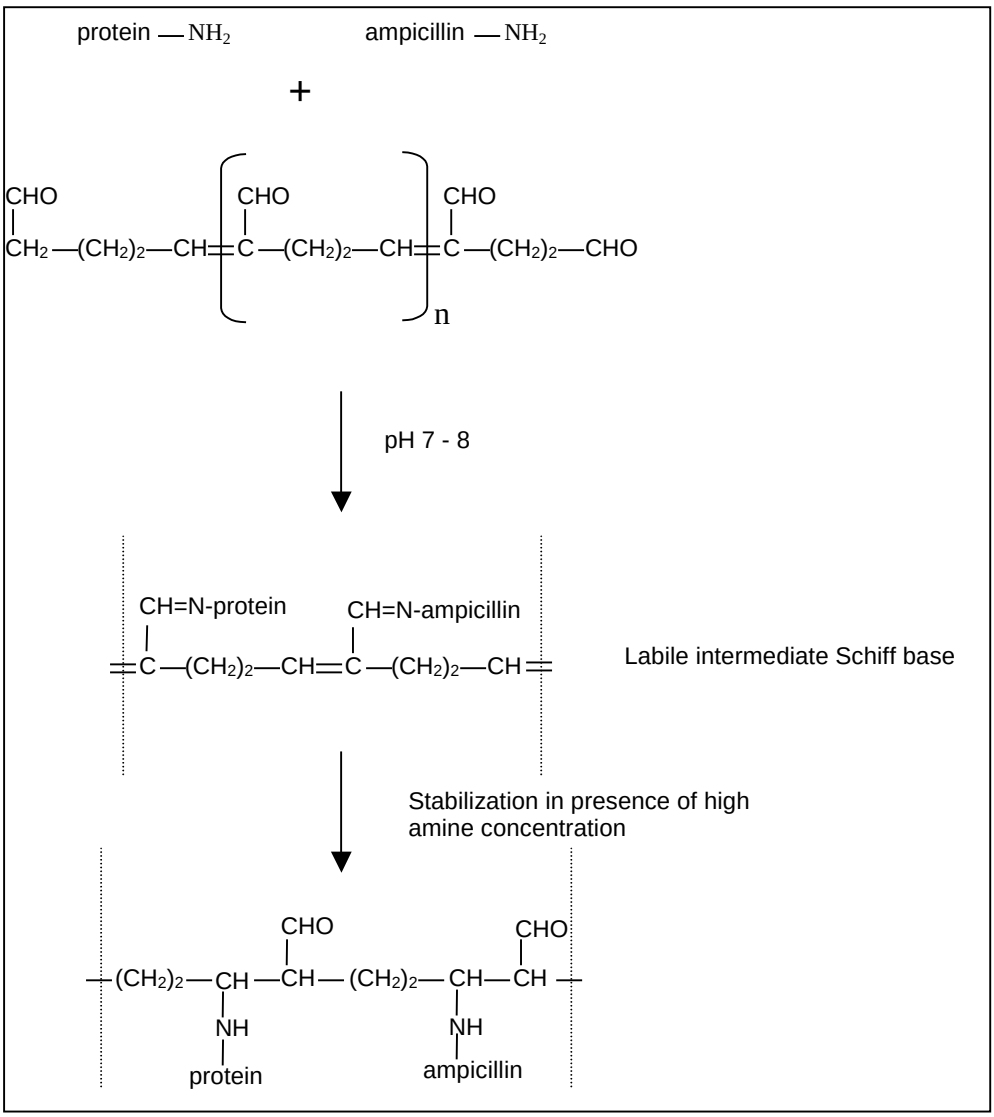


Figure 4.3:conjugation of ampicillin to proteins using glutaraldehyde

Preparation of physiological penicillin-carrier conjugate (Katsutani and Shionoya, 1993; figure 4.4)

Penicillins are considered to bind covalently with proteins and form penicilloyl-protein conjugates. An alkaline condition facilitates the reaction (Katsutani and Shionoya, 1993).

One gram ampicillin and 0.25 g carrier-protein were dissolved in 10 ml 0.008 M diethylbarbituric acid pH 8.5 and incubated for 24 hrs at 37 °C. Subsequently, the solution was dialyzed against PBS for 2 days. Aliquots of the conjugate phys amp-ova and phys amp-bsa were stored at –20 °C. The coupling efficiency was determined as done for the glutaraldehyde conjugates.

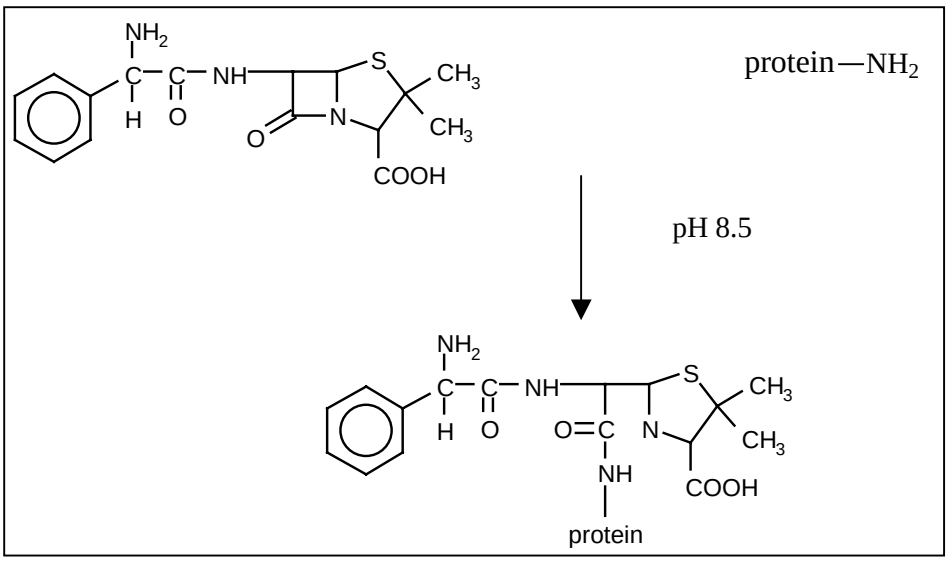


Figure 4.4: Physiological conjugation of ampicillin to proteins

Immunization and production of monoclonal antibodies (mAb)

Balb/c mice (minimum 10 weeks old) were immunized with different ampicillin-protein conjugates using 3 different immunization procedures. The first method consisted of an intraperitoneal injection of 50 µg of conjugate (emulsified in 100 µl sterile PBS and 100 µl CFA), followed 3 weeks later by a second (and third) intraperitoneal injection with the same amount of conjugate (emulsified in sterile PBS and IFA). The second method differed from the first method in that the second (and the third) injection was given after 4 weeks. In both methods, four or five days before fusion the mice with the highest specific serum antibody titres were injected intraperitoneally with 100 µg of conjugate in PBS. Blood samples were collected from the tail vein of the mice starting two weeks after the second immunization and tested in ELISA for the presence of penicillin-specific antibodies. In the third method both hind footpads of mice were injected with 100 µg conjugate (emulsified sterile PBS and IFA). Two weeks after this single injection the popliteal lymph nodes were used for fusion.

Hybridomas were obtained by polyethylene glycol mediated fusion or by electrofusion of SP2OAG/14 mouse myeloma cells with either spleen (method 1 and 2) or lymph node (footpad immunization) cells from immunized mice. The isolation of lymphocytes, the culturing of the myeloma cells and the polyethylene glycol mediated fusion was performed according to the procedures previously described by Harlow and Lane (1988*).* For the electrofusion, the myeloma cells and the lymphocytes were mixed at a ratio of 1:1, washed by centrifugation (5 min, 800 g, 18 °C) and resuspended in 300 µl sucrose containing buffer (0.255 M sucrose + 0.2 mM $CaCl_2$ + 0.2 mM $MgCl_2$ diluted in distilled water). The

suspension was transferred into the fusion chamber of the electroporesis apparatus (ICN Biomedicals, Asse-Relegem, Belgium). The fusion was performed as follows: 1) dielectrophoresis (frequency 1 MHz, field strength 15 V/ 0.5 cm, 30 sec), 2) electroporation (field strength 0.15 V/ 0.5 mm, 5 µsec, one pulse) 3) dielectrophoresis again. Finally, the cell suspension was removed from the fusion chamber, diluted in HAT-selection medium and distributed over 96-well tissue culture plates at 1000 to 10 000 cells per well. Hybridomas producing penicillin-specific antibodies were cloned twice by limiting dilution (Harlow and Lane, 1988).

Indirect ELISA

Microtiter plates were coated overnight at 4 °C with ampicillin-succinimide ester-ovalbumin conjugate (amp-MBS-ova; 100 µl/well) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). The plates were washed 3 times with PBS containing 0.05% Tween® 20 between each incubation step. Free binding sites were blocked with 200 µl of a glycine solution (5% glycine in coating buffer) for 2 hrs at 37 °C. Subsequently 100 µl/well of an appropriate dilution of mice sera or hybridoma supernatant in PBS containing 3% bsa and 0.05% Tween® 20, were added. The plates were incubated for 1 hr at 37 °C. Then, 100 µl/well of the secondary antibody (diluted in PBS containing 3% bsa and 0.05% Tween® 20) were added for 1 hr at 37 °C, where after 50 µl/well enzyme substrate ABTS solution were added. Subsequently the plates were incubated at 37 °C. The colour development was measured at 405 nm using an ELISA reader (Spectrafluor, TECAN) and presented as optical density (OD).

Competitive inhibition ELISA

The only difference between the competitive inhibition ELISA and the indirect ELISA was that in the former, the samples (mice sera and hybridoma supernatant) were preincubated at RT with a 10 mM ampicillin solution or with serial dilutions (concentrations ranging from 10 mM to 0.01 mM) of a β-lactam antibiotic mixture containing ampicillin, benzylpenicillin, carbenicillin, dicloxacillin and oxacillin. After 1 hr the antibody-antibiotic mixture was tested in ELISA.

The competition in the ELISA between a free penicillin in the sample and the coated ampicillin (amp-MBS-ova) was calculated with the formula: competition (%) = $(1-(A/A_o))*100$ with A= absorbance of a tested sample solution and A_o the absorbance of a similar solution without penicillin.

Results

Preparation of penicillin immunogens

Four coupling procedures were used to develop penicillin-carrier conjugates: the carbodiimide mediated coupling, glutaraldehyde, succinimide ester method and a physiological reaction method. Ampicillin was chosen as hapten and conjugated to bovine serum albumin (bsa), ovalbumin (ova) or thyroglobulin (thyro) using the 4 procedures (Table 4.1). Conjugation was performed in such a way that the common structure of penicillins, the thiazolidin ring, was left unchanged for the induction of common antibodies to β-lactam antibiotics. The chemical structures of the obtained immunogens are presented in the reaction schemes (figures 4.1, 4.2, 4.3, and 4.4). The efficiency of the coupling reaction was determined as the amount of ampicillin molecules bound to one carrier molecule. For each procedure, a coupling efficiency of approximately 10 (8 to 16) was obtained (Table 4.1), except for the carbodiimide where coupling efficiency could not be determined. When determining the amount of bound ampicillin in these carbodiimide-mediated conjugates, coupling efficiencies of more than 100 were obtained. Since the carrier protein and ampicillin were conjugated in a ratio protein:ampicillin of 1:100, and since no protein was removed from the reaction mixture, coupling efficiency cannot be higher than 100. The overestimation of the amount of bound ampicillin molecules was probably due to remaining citraconic anhydride molecules or carbodiimide molecules not removed by dialysis.

Table 4.1: Methods used for ampicillin-protein conjugation

Conjugation method	Coupling efficiency ($molec_{ampicillin}/molec_{carrier}$)	Determination method
Carbodiimide	ND[1]	/
Glutaraldehyde	8.5 – 16	BCA
Succinimide ester	8 – 13	Ellman standard method
Physiological	11 – 13	BCA

[1]ND: not determined

Antibody response

Blood samples were collected 2 weeks after each immunization and were tested for anti-ampicillin antibodies in the indirect and the inhibition ELISA (Table 4.2). No anti-ampicillin antibodies could be detected in the mice immunized with the carbodiimide

mediated ampicillin-albumin conjugates. Moderate antibody responses against ampicillin were obtained after immunization with the carbodiimide mediated ampicillin-thyroglobulin, the glutaraldehyde or succinimide conjugates. Subsequent immunizations did not enhance the response. A high antibody response was induced using the physiological ampicillin-protein conjugate. Using ova or bsa as the carrier protein, no difference in antibody response against the hapten could be observed when comparing immunogens differing only for their carrier protein.

Table 4.2: Immunogenicity of different ampicillin-protein conjugates

Conjugation method	Carrier-protein[1]	Immunizations		Number of mice with specific response in indirect ELISA	Indirect ELISA[3]	Inhibition ELISA[4]
		Route[2]	Number of mice			
Carbodiimide	bsa	IP1	3	0	-	ND[5]
	ova	IP1	3	0	-	ND[5]
	thyro	IP1	3	3	+/+++	ND[5]
Glutaraldehyde	bsa	IP1	3	3	+/++ +	0/++
	ova	IP1	3	3	+/+++	0/++
Succinimide	bsa	IP1	3	3	++	+/++
Physiological	bsa	IP2	4	4	++++	++++

[1]bsa = bovine serum albumin, ova = chicken egg albumin, thyro = thyroglobulin
[2]IP1 = intraperitoneal, interval between subsequent immunization = 3 weeks; IP2= intraperitoneal, interval between subsequent immunization = 4 weeks;
[3]Scores are given according to O.D. levels obtained with 1/20 diluted sera: - = < 0,200; + = 0.200 - 0.500; ++ = 0.500 - 1.000; +++ = 1.000 - 1.800; ++++ = > 1.800.
[4]Scores are given according to the obtained inhibition of antibody binding by preincubation with 10 mM ampicillin: + < 50 %; ++ = 50-75%; +++ = 75-90%; ++++ >90%
(The percentage of inhibition = 100 - [OD-value inhibition ELISA/ OD-value indirect ELISA] x 100)
[5]ND: not determined

Production of mAb

An overview of the fusion experiments is given in Table 4.3 for mouse 4 immunized intraperitoneally (IP) with carbo amp-thyro, mouse 15 immunized IP with amp-glut-bsa, mouse 24 immunized IP with amp-MBS-bsa, mouse 35 immunized in the footpad (FP) with phys amp-ova and mouse 36 immunized FP with amp-MBS-ova. The footpad procedure resulted in the collection of only 1 to 2 x 10^7 cells from the popliteal lymph nodes and in the generation of few hybridomas, of which two with low affinity for penicillin: more than 10

mM ampicillin was needed to obtain 50% competition in the inhibition ELISA (Table 4.3). The IP immunization with carbodiimide mediated thyroglobulin conjugate, glutaraldehyde or succinimide-conjugates followed by fusion of spleen lymphocytes resulted in at least 10 times higher number of hybridomas. However, again no or only few hybridomas produced penicillin-specific antibodies which had moreover a low affinity as presented in Table 4.3 for mouse 4, 15 and 24.

Four mice immunized intraperitoneally with a physiological conjugate, were selected for spleen cell fusion experiments (mouse 161 to 164, Tables 4.4 en 4.5). This selection was based upon either a high serum antibody titer (mouse 163 and 164) in the indirect ELISA, or a strong competition effect in the competitive inhibition ELISA (mouse 161 and 162).

The immunization frequency, the injection method at the final booster injection, the detection limit of diluted serum in the indirect ELISA and the competition effect are represented in Table 4.4, while data obtained after cell fusions are given in Tables 4.5 and 4.6. Fusion experiments with mouse 162 and 164 resulted in several hybridomas showing strong competition effect (Table 4.5). Unfortunately, the hybridomas derived from mouse 162 were not stable.

The most important finding was that the best hybridomas were obtained after spleen cell fusion of a mouse (mouse 164) with an intravenous final booster, whereas the other mice got an intraperitoneal final booster injection.

Table 4.3: Overview of the data obtained after fusion experiments

Mouse n°	Immunogen	Route[1]	Fusion procedure	Number of hybridomas	Number positives in indirect ELISA	Number positives[2] in inhibition ELISA
4	carbo amp-thyro	IP	PEG mediated	313	48	0
15	amp-glut-bsa	IP	PEG mediated	81	35	1
24	amp-MBS-bsa	IP	Electrofusion	214	2	1
35	phys Amp-ova	IFP	Electrofusion	8	2	2
36	amp-MBS-ova	IFP	PEG mediated	4	0	0

[1]IP1 = intraperitoneal, interval between subsequent immunization = 3 weeks; IFP = footpad
[2]Binding of antibodies to the coated ampicillin-carrier conjugate could be partially inhibited by preincubation with a 10 mM ampicillin solution (= 3.71 mg/ml).

Table 4.4: Analysis of sera of mice immunized intraperitoneally, with the physiological conjugate

Mouse n°	Number of immunizations	Final booster[1]	Antibody titer in indirect ELISA	Inhibition ELISA[2]
161	2	IP	1000	++
162	2	IP	1000	++++
163	5	IP	8000	+
164	4	IV	10 000	+

[1]IP = intraperitoneal, IV = intravenous
[2]Scores are given according to the obtained inhibition of antibody binding by preincubation with 10 mM ampicillin: + < 50 %; ++ = 50-75%; +++ = 75-90%; ++++ >90% (The percentage of inhibition = 100 - [OD-value inhibition ELISA/ OD-value indirect ELISA] x 100)

Table 4.5: Analysis of hybridomas of mice immunized with the physiological conjugate

Mouse n°	Total number	Number positives in indirect ELISA	Number positives[1] in inhibition ELISA	Number with inhibition > 70%
161	> 3000	95	15	0
162	> 4000	90	37	(7)
163	+/- 3000	321	10	0
164	1417	180	32	21

[1]Binding of antibodies to the coated ampicillin-carrier conjugate could be inhibited partially or completely by preincubation with a 10 mM (3.71 mg/ml) ampicillin solution

Table 4.6: Characteristics of the selected mAb, derived from mouse n° 161 and 164

Mouse n°	mAb	Isotype	% inhibition[1] with ampicillin			Cross-reaction[2] with 1 µg/ml		
			5 mg/ml	100 µg/ml	100 ng/ml	penG	carb	Oxa
161	12F6	IgM	22	10	0	-	-	-
161	12F5	IgM	20	11	0	-	-	-
161	13B2	IgM	26	12	0	-	-	-
164	10E5	IgG1	100	100	10	+	+	+
164	19C9	IgG1	100	100	13	+	+	+
164	10C2	IgG2a	100	100	0	+	+	+
164	23D12	IgM	100	75	0	-	+	+
164	2G4	IgG2a	100	75	0	+	-	-
164	9H3	NT[3]	100	78	0	+	+	+

[1]% inhibition was determined in the inhibition ELISA. Therefore the monoclonal was preincubated during 30 min. with native ampicillin.
[2]Cross-reactivity was examined in the inhibition ELISA. penG = penicillin G; carb = carbenicillin; oxa = oxacillin
[3]NT = not tested

Hybridomas selected for further study

After subcloning and ascites production the isotype and cross-reactivity with other penicillins were investigated. In table 4.6 these characteristics are represented for mAbs derived from two mice: mouse 161 and mouse 164. As can be seen, mAbs derived from mouse 164 showed a higher percentage of inhibition than the mAbs derived from mouse 161. Binding of two of the mAbs (10E5, 19C9) of mouse 164 to an ampicillin-carrier conjugate could even be partially inhibited by preincubation with ampicillin at a concentration of 100 ng/ml. Results therefore indicate that the affinity of the antibodies from mouse 164 for native ampicillin is high. Furthermore, 4 of them (19C9, 10E5, 10C2 and 9H3) cross-reacted with other penicillins, suggesting that they recognized common epitopes.

For the determination of cross-reactivity of the mAb 19C9 for several penicillins at their MRL value (Table 4.7), the competitive inhibition ELISA was performed at two different incubation temperature (4 °C and 37 °C) and two different incubation times (30 min and 60 min). The best results for the detection of ampicillin and penicillin G were obtained when the ELISA was incubated during 30 min at 4 °C. Otherwise, the detection of oxacillin, carbenicillin and dicloxacillin was more sensitive at 37 °C. Cross-reaction of this antibody with sulfanilamide, chloramphenicol, neomycin and streptomycin was not observed.

Table 4.7: Cross-reactivity (at Maximum Residue Level, MRL) of mAb 19C9 with other penicillins and some other antibiotics and sulfanilamide, measured in the inhibition ELISA

Molecules	Concentration[1] (ng/ml)	% inhibition	MRL detection level?	Incubation conditions on ELISA-plate
Ampicillin	50	31	Yes	4 °C, 30 min.
Penicillin G	50	25	Yes	4 °C, 30 min.
Carbenicillin	50	25	Yes	37 °C, 15 min.
Oxacillin	300	40	Yes	37 °C, 60 min.
Dicloxacillin	300	40	Yes	37 °C, 60 min.
Sulfanilamide	500	0		4 and 37 °C, 15 and 30 min.
Chloramphenicol	500	0		4 and 37 °C, 15 and 30 min.
Neomycin	500	0		4 and 37 °C, 15 and 30 min.
Streptomycin	500	0		4 and 37 °C, 15 and 30 min.

[1]MRL maximum residue level

Discussion

The carrier-hapten conjugates used for immunization in the present study were characterized by determining the number of ampicillin molecules per carrier molecule and by their capacity to induce polyclonal antibody responses in mice. For the carbodiimide mediated conjugates, no appropriate method was available for determining the number of ampicillin molecules per carrier molecule. Since these conjugates were found to be poorly immunogenic, no further research was done for the characterisation of these conjugates. For the other conjugates, the number of ampicillin molecules coupled to one molecule protein could be measured, and was similar to or slightly higher than these obtained by other investigators (Van Regenmortel et al., 1988; Märtlbauer, 1993; Katsutani and Shionoya, 1993). Katsutani and Shionoya (1993) constructed physiological benzylpenicillin-bsa and -ova conjugates with a hapten/carrier coupling efficiency of 18/1 and 10/1 respectively. Märtlbauer (1993) used the glutaraldehyde method to obtain sulfonamide-carrier conjugates with a coupling efficiency of 7/1, and the active ester method (succinimide) for coupling natamycin to a carrier with a coupling efficiency of 5/1. Van Regenmortel et al. (1993) stated that a coupling efficiency of 5 to 20 mol hapten per mol carrier was high enough to render the hapten immunogenic. This was consistent with findings in the present study, as most immunizations induced antibody responses. However, the antibody response against non-physiological conjugates was moderate to low. Also, Usleber et al. (2000) immunized with ampicillin coupled to different carriers using glutaraldehyde as cross-linker and found the conjugates being non- or weakly immunogenic.

Immunizations with the carbodiimide-mediated ampicillin-albumin conjugates did not induce anti-ampicillin antibodies whereas antibodies against the carrier-protein could be demonstrated. However, when thyroglobulin was used as the carrier-protein, an ampicillin-specific response was induced. Because thyroglobulin is at least ten times larger than albumin, the lack of antibody response to the carbodiimide mediated ampicillin-albumin conjugate suggests that albumin did not carry as many ampicillin molecules as thyroglobulin.

Using the immunogens constructed with cross-linker or the carbodiimide mediated conjugate, few hybridomas were obtained. Only some of them produced low affinity penicillin-specific antibodies (Table 4.3). This indicates that these immunogens or the

immunization procedures were unfavorable. Immunization using the footpad method resulted in few hybridomas compared to spleen cell fusion experiments. Consequently, lower number penicillin-specific hybridomas were obtained (mouse 35 and 36; Table 4.3). Interestingly, the two hybridomas of mouse 35, which were found positive in the indirect ELISA, were also positive in the inhibition ELISA. Fusion experiments following intraperitoneal immunization always resulted in a higher amount of hybridomas, but only very few were found positive in the inhibition ELISA (Table 4.3, mouse 4, 15, 24). Mirza et al. (1987) used human insulin as immunogen to compare different routes of immunization for hybridoma production. They found that footpad immunization followed by popliteal lymph node lymphocyte fusion yielded 100% of the hybridomas secreting specific antibody, compared to subcutaneous or intraperitoneal immunization followed by splenic lymphocyte fusion (8%). However, another study using the footpad immunization procedure for the production of antibodies against viral and bacterial antigens, resulted in 6 to 28% hybridomas secreting specific antibodies (Coyle et al., 1992). A disadvantage of the footpad immunization is the low number of lymphocytes that can be used for cell fusion. Furthermore, this kind of immunization is very painful and should therefore only be used if really necessary (Harlow and Lane, 1988).

In this study two methods were used for fusion: electrofusion and polyethylene glycol (PEG) mediated fusion. Electrofusion is widely described to yield higher fusion efficiency with more antigen-specific hybridomas than the PEG mediated fusion (Karsten et al., 1988; Harlow and Lane, 1988; van Duijn et al., 1989). However, in the present study no difference could be observed between both methods.

A high antibody response was obtained against the physiological ampicillin-protein conjugates. Similar conjugates are formed in vivo following penicillin administration (Katsutani and Shionoya, 1993). Natural conjugation results in an open β-lactam ring structure with loss of antimicrobial activity, but not immunogenicity (Dewdney et al., 1991). The mAbs obtained from mice immunized with physiological conjugates showed strong competition in the inhibition ELISA. The antibodies could partially be inhibited for binding to the coated ampicillin-carrier conjugate after preincubation of these antibodies with ampicillin at a concentration of 50 ng/ml.

From the results it appears that the route of antigen administration during the final booster injection could be very important. The mAbs derived from mouse 164, which was

boosted intravenously, recognize penicillin better than these obtained from mouse 161, which was boosted intraperitoneally. Intravenous injection will result in a rapid and strong response of splenic lymphocytes, as the antigen will be captured quickly in the spleen (Harlow and Lane, 1988). So, directing the antigen during the final boost toward the lymphocytes that will be used in the fusion seems to increase the number of antigen-specific antibodies. Monoclonal 19C9 displayed a specific cross-reactivity, as defined in the inhibition ELISA, with ampicillin, penicillin G, oxacillin, dicloxacillin and carbenicillin, and not with sulfanilamide, chloramphenicol, neomycin and streptomycin. Consequently, mAb 19C9 appears a β-lactam-specific antibody and looks very promising for developing an ELISA able to detect most penicillins at their MRL concentration.

Acknowledgements: This study is supported by the Ministry of Agriculture, Brussels, Belgium and by the Institute for Veterinary Inspection (IVK). The technical assistance of Els De Vogelaere was greatly appreciated.

Chapter 5

Production of penicillin-specific polyclonal antibodies for a class-specific screening ELISA

Based on: P. Cliquet, B. M. Goddeeris and E. Cox. Production of penicillin-specific polyclonal antibodies for a class-specific screening ELISA. Journal of Agricultural and Food Chemistry, submitted

Abstract

Polyclonal penicillin-specific antibodies were obtained after immunization of rabbits (K2, K6 and K8) with physiological ampicillin- and benzylpenicillin-protein conjugates (pAb K2) or with physiological ampicillin-, benzylpenicillin-, oxacillin and dicloxacillin-protein conjugates (pAb K6 and pAb K8). The broad-specificity of the antisera induced by physiological penicillin-protein conjugates was improved by alternately immunization with different penicillins as hapten. With each of the polyclonals, an antigen coated and an antibody coated competitive inhibition (ci)ELISA was constructed. For the three antisera, the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin was more sensitive in the antibody ciELISA as compared to the antigen ciELISA. The detection of all penicillins in buffer solutions below the MRL in the antibody ciELISA was achieved when the penicillins were hydrolysed with Penicillinase I.

Keywords: Penicillin – polyclonal antibodies – ELISA

Introduction

Penicillins are frequently used in veterinary medicine to treat and prevent bacterial infections. They are also used as feeder additive to increase feed efficiency, promote growth and prevent disease (Boison, 1995). Consequently, residues can be found in food derived from treated animals and harm the health of consumers (Milhaud and Person, 1981). Microbiological and immunological assays are usually used to screen food products for the presence of anti-microbial drug residues (Allison, 1985; Charm and Chi, 1988; Kavanagh, 1989). Microbiological assays do not differentiate between families of antimicrobial drugs whereas immunological assays allow the group-specific detection. For the development of a penicillin-specific immunological assay, monoclonal or polyclonal antibodies against the common penicillin structure are required.

Monoclonal antibodies bind to just one epitope of a molecule and are therefore very specific, have a high degree of purity, are easily reproducible and are therefore very suitable for the development and standardisation of a test system. In contrast, large variations can occur during the production of polyclonal antibodies. On the other hand, polyclonals are less influenced by physiological parameters (pH, temperature, …) and have a higher avidity as compared to monoclonals (Cambell, 1984; Tijssen, 1985; Booman, 1988). In ELISA,

antibodies with high affinity or avidity are required because low affinity interactions are rapidly dissociated during the wash steps. As a result, it is possible that an ELISA using polyclonal antibodies is more sensitive than an ELISA using monoclonals (Tijssen, 1985).

The aim of our research was to develop a penicillin-specific ELISA. Therefore, monoclonal (Cliquet et al., 2001) and polyclonal antibodies against the common penicillin structure were developed. This article describes the production and analysis of the polyclonals.

The production of a sensitive penicillin-specific antiserum is difficult. Different chemical methods have been reported to couple ampicillin to the carrier protein via the amino group to synthesize immunogens, which are able to induce antibodies against the intact penicillin structure (Kitagawa et al., 1978; Nagakura et al., 1991; Usleber et al., 1998). Only Usleber and coworkers (1998) obtained a rabbit antiserum for which an ELISA could be developed able to detect several penicillins at concentrations in the range of 5 to 20 ng/ml. Using 6-aminopenicillanic acid as hapten for the development of a penicillin-protein conjugate with intact β-lactam structure provided weak immunogens (De Leuw et al., 1997). Other attemps to produce group-specific antisera using as immunogen β-lactam coupled to the carrier protein through the carboxy-group led to highly specific antisera but again not sensitive enough (Kachab et al., 1992; Usleber et al., 1994). Direct covalent binding of the penicillin to the carrier protein (physiological conjugation) led to antisera highly reactive for the open β-lactam ring structure and less against the native molecule (Rohner et al., 1985; Usleber et al., 1998; Grubelnik et al., 2001). The commercial immunoassay, LacTek™ ELISA for the detection of penicillins in the range of the MRL (Idetek, Mitchell et al., 1999) is not available anymore and is replaced by the Parallux™ assay (Idexx Laboratories). The Parallux™ assay is developed for the detection of ampicillin, amoxicillin and benzylpenicillin in milk (Huth et al., 2002) but can also be applied for the detection of these pencillins at the MRL in bovine and porcine kidney tissues (Okerman et al., 2003). Unfortunately, no details are published on the production and characterization of the antibodies used in these assays.

In the present study, polyclonal rabbit antibodies against the group of penicillins were produced using physiological penicillin-carrier protein conjugates as immunogens. The production of these conjugates has already been described (Cliquet et al., 2001). To improve the broad-specificity of the antisera, immunogens with different penicillins were used for subsequent immunization. The antisera were analysed in an antigen and in an antibody competitive inhibition (ci) ELISA for detection of different penicillins.

Material and methods

Reagents and chemicals

Benzylpenicillin, oxacillin, dicloxacillin, cefaclor, cephadrin, cephalexin, cefazolin, sulfanilamide, chloramphenicol, bovine serum albumin (bsa), kaolin (hydrated aluminum silicate), Penicillinase (EC 3.5.2.6, type 1, from *B. cereus*) and 3, 3', 5, 5'-tetramethylbenzidin (TMB) were purchased from Sigma Chemicals (Bornem, Belgium). The TMB enzyme-substrate solution was prepared by adding 100 µl of a TMB stock solution (10 mg TMB dissolved in 1 ml dimethylsulfoxide) to 10 ml phosphate-citrate buffer pH 5 (25.7 ml 0.2 M Na_2HPO_4 + 24.3 ml 0.1 M citrate + 50 ml distilled water) supplemented with 1.3 µl H_2O_2. Amoxicillin and cloxacillin were from ICN Biochemicals (Asse-Relegem, Belgium) and ampicillin and ABTS® (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Tablet and buffer) from Roche Diagnostics (Brussels, Belgium). The ABTS enzyme-substrate solution was prepared by dissolving 1 ABTS tablet® (50 mg) in 50 ml ABTS buffer®. Tween® 20 (polyoxyethylene sorbitan monolaurate) was purchased from Merck-Belgolabo (Overijse, Belgium). Peroxidase-labelled swine anti-rabbit immunoglobulins (code n° P0217) were from DAKO Diagnostica (Prosan, Ghent, Belgium). EZ-link™ Sulfo-NHS-LC-Biotin was purchased from Pierce (Perbio, Erembodegem-Aalst, Belgium). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were provided by Difco Laboratories, Biotrading (Bierbeek, Belgium). ELISA microtiter plates (Maxisorp®) were provided by NUNC (VWR international, Leuven, Belgium). All other chemicals were of reagent grade.

The ELISA coating antigen, an ampicillin-ovalbumin conjugate (ampMBSova), was synthesized with an activated ester method using the hetero-bifunctional reagent *m*-maleimido benzoyl-*N*-hydroxisuccinimide ester (MBS) (Cliquet et al., 2001).

Production of polyclonal antibodies

New Zealand white rabbits were immunized subcutaneously with 500 µg of the physiological penicillin-carrier conjugate (Cliquet et al., 2001). For the first immunization, the conjugate was emulsified in 500 µl sterile saline and 500 µl complete Freund's adjuvant. For all subsequent immunizations, at six weeks interval, incomplete Freund's adjuvant was used. Blood was sampled two weeks after each immunization. The serum was collected (3000 g, 20 min) and treated with kaolin (Van den Broeck et al., 1999). Therefore one part of serum was mixed with four parts of kaolin and incubated for 30 min at room temperature. The

mixture was centrifuged (3000 g, 20 min) and the supernatant was stored frozen (-20°C). The polyclonal antibodies (pAb) were analysed in ciELISAs.

Biotinylation of penicillin

Ampicillin or amoxicillin (1.2 mg) were diluted in 300 µl phosphate buffer 0.1 M, pH 7.2 and added to 3.7 mg of a succinimide ester-biotin conjugate (EZ-link™ Sulfo-NHS-LC-Biotin) diluted in 700 µl phosphate buffer 0.1 M, pH 7.2. The reaction mixture was incubated for 24 hours at room temperature while shaking. It was not necessary to remove the unreacted biotin: the conjugate was diluted at least 1000 times in ELISA. At this dilution no background signals were observed. Aliquots of the conjugate were stored at –20 °C.

Competitive inhibition ELISA coated with antigen (antigen ciELISA)

Microtiter plates were coated overnight with 2.5 µg/ml ampMBSova (Cliquet et al., 2001) diluted in bicarbonate coating buffer (0.05 M; pH 9.4) followed by blocking for two hours at 37 °C with 5% glycin in coating buffer. Subsequently, a penicillin was added (100 µl/well) diluted in PBS (0.15 M; pH 7.4) containing 3% bsa and 0.05% Tween® 20 (dilution buffer) together with an appropriate concentration of the penicillin-specific polyclonal antibodies pAb K2, pAB K6 or pAb K8 (100 µl/well). The microtiter plates were then incubated for one hour at 37 °C. Next, the plates were incubated with peroxidase labelled swine anti-rabbit antibodies in dilution buffer for 60 min at 37 °C. Between each step, the plates were washed three times with PBS containing 0.05 % Tween® 20. Finally, the plates were incubated for one hour at 37 °C with the enzyme substrate solution (ABTS, 50 µl/well). The color development (absorbance (A) or optical density (OD)) was measured at 405 nm using an ELISA reader (Spectrofluor, TECAN).

Competitive inhibition (ci)ELISA coated with antibody (antibody ciELISA)

Microtiter plates were coated overnight with pAb K2, pAB K6 or pAb K8 (1/1000) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). After coating, the plates were blocked for two hours at 37 °C with 0.2 % Tween®80 in coating buffer. Subsequently, a penicillin was added in dilution buffer, as for the antigen ciELISA. The plates were then incubated for one hour at 4 °C. Next, 100 µl of biotinylated ampicillin (for pAb K2 and pAb K6) or biotinylated amoxicillin (for pAb K8) in dilution buffer was added, without washing the plate, for 30 min at 4 °C. The other wash steps occurred as for the antigen ciELISA. Then, the plates were

incubated for 30 min at 37 °C with streptavidin-peroxidase conjugate in dilution buffer. Finally, the enzyme substrate solution (TMB) was added to the plates for one hour at 37 °C. The color development was measured at 650 nm.

Interpretation of the ELISA results

The competition in the antibody ciELISA between a free penicillin in the sample and the biotinylated penicillin was calculated with the same formula as in the antigen ciELISA: competition (%) = (1- (A/A_o))*100 with A = absorbance of a tested sample solution and A_o the absorbance of a similar solution without free penicillin.

The calibration curve was obtained by plotting the concentration of a standard dilution of penicillin against the absorbance measured for the binding of the antibodies (in the antigen ciELISA) or biotinylated penicillin (in the antibody ciELISA) in the presence of standard dilutions.

The limit of detection (LOD = average signal – 3* SD) and IC50-value (concentration of free penicillin for which the binding of the antibodies is inhibited for 50 %) in ng/ml are obtained by extrapolation of the response or absorbance in a calibration curve.

Hydrolysis of the sample penicillin

Without hydrolysis: penicillin was diluted in PBS just before use, to avoid spontaneous hydrolysis.

Spontaneous hydrolysis: penicillin was diluted in PBS and incubated overnight at room temperature.

Enzyme mediated hydrolysis: Penicillinase was diluted in PBS (1000 unit/ml) and stored at –80°C. Prior to use, the penicillinase I stock was further diluted (0.1 unit/ml). Five microliter of this penicillinase dilution was added to 1 ml sample. Next, the mixture was incubated during one hour at 37°C followed by a 30 min incubation at 4°C to inactivate the enzyme (Medina et al., 1998).

Results and discussion

Development of penicillin-specific antibodies and an antigen ciELISA

The polyclonal antisera (pAb) were obtained after immunization of rabbits (K2, K6, K8) with physiological ampicillin- and benzylpenicillin-protein conjugates (pAb K2) or with physiological ampicillin-, benzylpenicillin-, oxacillin and dicloxacillin-protein conjugates (pAb K6 and pAb K8).

Based on the allergenicity of penicillins, no differences in immunogenicity should be observed when using different penicillins as hapten. The β-lactam ring of all penicillins spontaneously opens under physiological conditions, forming the penicilloyl group. This group is designated as the major determinant because approximately 95% of the penicillin molecules that irreversibly combine with proteins form penicilloyl moieties. Penicillins are also degraded by other metabolic pathways to form, in smaller quantities, other antigenic derivatives, called minor determinants. Both major and minor determinants can bind to proteins and elicit an immune reaction (Chowdhury and Lieberman, 1999). Thus, a strong immune response should be elicited with physiological penicillin-protein conjugates, independent of the penicillin used as hapten (Katsutani and Shionoya, 1993). This was in accordance with our results since high serum antibody titers were obtained after immunization of rabbits and mice (Cliquet et al., 2001).

Patients allergic to any member of the penicillins should be considered allergic to all of them (Raynor, 1997). Thus, cross-reactivity for the other members of the penicillin family was expected and also obtained. The IC50-values of the three antisera for six penicillins analysed in the antigen ciELISA are shown in table 5.1. Although four different conjugates were used for the immunization of K6 and K8 in contrast to only two different immunogens for K2, the same pattern of recognition was obtained for pAb K2 and pAb K8 and a slightly different pattern for pAb K6 (Table 5.1). Ampicillin and benzylpenicillin are best recognized by pAb K2 and pAb K8, and ampicillin also by pAb K6. Amoxicillin is also very well recognized by pAb K2 and pAB K8, but not by pAb K6 (which recognizes amoxicillin the worst). The antiserum pAb K6 is more sensitive to cloxacillin and dicloxacillin as compared to pAb K8, despite the fact that both antisera were obtained after immunization with oxacillin and dicloxacillin-protein conjugates. Even pAb K2 is more sensitive for these penicillins than pAb K8. From the specificity of the three antisera, it could be concluded that it is sufficient to immunize with ampicillin- and benzylpenicillin-protein conjugates for the induction of penicillin-specific antibodies, except for the dicloxacillin recognition.

However, to deduce scientifically and statistically which conjugate is the most appropriated for the development of penicillin-specific polyclonal antibodies more than three rabbits should be immunized. And even so, it is well known that the immune response to the same immunogen can vary between animals.

Table 5.1: IC50-values (ng/ml) for 6 penicillins in buffer, not hydrolysed and analysed in the antigen ciELISA with polyclonal antibodies pAb K2, pAb K6 and pAb K8.

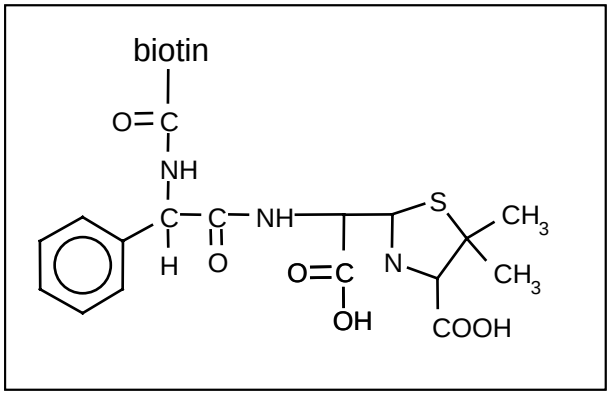
Polyclonal	Hydrolysis	IC50-values (ng/ml) for penicillins[1] in the antigen ciELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	no	1000	6000	1800	10000	8000	20000
pAb K8	no	400	4000	3000	10000	10000	30000
pAb K6	no	200	30000	9000	15000	8000	8000

[1] amp = ampicillin; amox = amoxicillin; bpg = benzylpenicillin; ox = oxacillin; clox = cloxacillin; diclox = dicloxacillin.

Development of an antibody ciELISA

Biotin was conjugated to the side chain of ampicillin (Figure 5.1) and amoxicillin, leaving the common penicillin structure free for antibody binding. These conjugates allowed the development of an antibody ciELISA with the three antisera. For all pAbs, the detection of penicillins is more sensitive in the antibody ciELISA as compared to the antigen ciELISA (Table 5.3 and 5.4, no hydrolysis).

Figure 5.1: Biotinylated ampicillin



The difference in sensitivity between both ELISAs could be ascribed to a combination of factors such as the use of a different competitor molecule (Choi et al., 2002), the immunogen used to produce the antibodies (Kirkley et al., 2001) as well as the set-up of the test system:

In the antigen ciELISA, the competition occurs between the penicillin in the sample and the coated ampicillin-ovalbumin conjugate whereas in the antibody ciELISA, biotinylated

ampicillin is used as competitor. In both cases, ampicillin is linked via its free amino group at the side chain of the molecule to biotin or to ovalbumin using the same coupling reaction between ampicillin and the linker molecule (succinimide ester) but using a different cross linker molecule (succinimide ester resp. maleimide ester). Moreover, the ampicillin coupled to biotin has more freedom of movement than the ampicillin coupled to ovalbumin because of the longer spacer arm of the linker molecule (length spacer arm of the succinimide ester = 22.4Å *vs* maleimide ester = 11.6 Å) and the smaller size of the linked molecule (MW biotin <1kD *vs* MW ovalbumin = 45 kD). Kirkley and coworkers (2001) demonstrated the influence of the conjugation method for hapten-carrier linkage on the immune response, and thus on the antibody binding. We made the same observation during the development of sulfonamide-specific antibodies (Cliquet et al., 2003). Our penicillin-specific antibodies are induced using physiologically linked penicillin-protein conjugates. No spacer arm at all is involved, thus resulting in a rather constrained conformation. The antibodies therefore probably have higher affinity for the ampicillin-ovalbumin conjugate as compared to the ampicillin, decreasing the sensitivity of the ELISA. Due to the longer spacer arm in the ampicillin-biotin conjugate in combination with the small size of biotin, the antibodies probably make no or less difference between the biotinylated ampicillin and the ampicillin in the sample.

In the antibody ciELISA, the antibodies are immobilized and the sample is incubated in the plate for one hour whereafter the biotinylated penicillin is added. The penicillins in the sample can thus bind to the coated antibodies before the biotinylated penicillin. In ELISA, dissociation rates are sometimes so low that the reactions can be considered practically irreversible. Consequently, the equilibrium may not be reached within the usual incubation time in ELISA (Van Regenmortel and Azimzadeh, 1994). This means that the penicillins bound to the coated antibodies will not dissociate during the assay incubation, inhibiting the binding of the biotinylated penicillin to form complexes with the coated antibodies.

In the antigen ciELISA, the antibodies preferentially bind to the coated ampicillin. The binding of antibodies to the coated ampicillin is probably favoured above the binding to free penicillins due to the higher affinity of the antibodies for a more constrained conformation of ampicillin. However, in the antibody ciELISA, the binding of the sample penicillins is favoured above the binding of the biotinylated penicillins due to the preincubation of sample and coated antibodies. As a result, higher sensitivities can be measured in the antibody ciELISA as compared to the antigen ciELISA.

Specificity of the polyclonals

The polyclonal antibodies pAb K2, pAb K6 and pAb K8 are highly specific for penicillins. No cross-reactions were observed for cephadrin, cephalexin, cefazolin, clavulanic acid, sulfanilamide or chloramphenicol (50, 500 and 5000 ppb) analysed in the antibody ciELISA. Cross-reactivities were only noticed for cefaclor when analysed at high concentration as compared to the penicillins (IC50 cefaclor approx. 5000 ppb as compared to IC50 < 200 ppb for all tested penicillins, table 5.2). It is not clear why the antibodies recognize cefaclor but not cephalexin or cephadrin. These three cephalosporins mainly differ in the substitution on the dihydrothiazin ring (-Cl for cefaclor *vs* $-CH_3$ for cephalexin and cephadrin). Probably the polyclonals mainly recognize the upper part of the thiazolidin and dihydrothiazin ring, since they do not recognize clavulanic acid (oxygen instead of sulphur). Other cephalosporins have larger side groups causing steric hindrance to the antibodies for binding the upper part of the dihydrothiazin ring.

Table 5.2: IC50-values (ng/ml) for 6 penicillins in buffer without hydrolysis, spontaneously hydrolysed and hydrolysed with an enzyme, and analysed in the antibody ciELISA with pAb K2, pAb K6 and pAb K8.

Polyclonal	Hydrolysis	IC50-values (ng/ml) for penicillins[1] in the antibody ciELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	no	55	150	450	150	800	>1000
	spontaneous	2	40	150	150	500	>1000
	enzyme	0.08	2	7	6	50	200
pAb K8	no	20	100	60	>1000	>1000	650
	spontaneous	15	80	50	300	1000	500
	enzyme	2	3.5	0.8	10	6	10
pAb K6	no	2.5	500	250	>1000	>1000	>1000
	spontaneous	2.5	300	250	>1000	>1000	>1000
	enzyme	1.5	150	40	60	200	150

[1] amp = ampicillin; amox = amoxicillin; bpg = benzylpenicillin; ox = oxacillin; clox = cloxacillin; diclox = dicloxacillin.

Table 5.3: Detection limit (LOD, ng/ml) for 6 non-hydrolysed penicillins in buffer, analysed in the antigen ciELISA with pAb K2, pAb K6 and pAb K8.

Polyclonal	Hydrolysis	LOD (ng/ml) for penicillins[1] in the antigen ciELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	no	6	200	150	500	550	1050
pAb K8	no	20	300	60	200	500	2000
pAb K6	no	20	2000	300	700	200	200

[1] amp = ampicillin; amox = amoxicillin; bpg = benzylpenicillin; ox = oxacillin; clox = cloxacillin; diclox = dicloxacillin.

Table 5.4: Detection limit (LOD, ng/ml) for 6 penicillins in buffer without hydrolysis, spontaneously hydrolysed and hydrolysed with an enzyme, and analysed in the antibody ciELISA with pAb K2, pAb K6 en pAb K8.

Polyclonal	Hydrolysis	LOD (ng/ml) for penicillins[1] in the antibody ciELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	no	1	10	30	7	60	200
	spontaneous	0.3	3	20	7	50	200
	enzyme	< 0.1	< 1	1	0.6	7	30
pAb K8	no	1.5	15	5	150	80	90
	spontaneous	1.5	8	5	50	60	90
	enzyme	< 1	< 1	< 1	< 1	< 1	1
pAb K6	no	0.3	50	20	100	200	200
	spontaneous	0.1	25	20	100	200	200
	enzyme	< 0.1	7	2	2	10	10

[1] amp = ampicillin; amox = amoxicillin; bpg = benzylpenicillin; ox = oxacillin; clox = cloxacillin; diclox = dicloxacillin.

Improving the sensitivity of the antibody ciELISA by hydrolysis of the test penicillin

The antigen ciELISA as well as the antibody ciELISA using the penicillin-specific polyclonal antisera are still not sensitive enough to detect the penicillins at the MRL in meat samples. The MRL in meat and meat products is 50 ppb for ampicillin, benzylpenicillin and amoxicillin, and 300 ppb for oxacillin, cloxacillin and dicloxacillin (Anonymous, 1990). Taking into account that the penicillin concentration is usually diluted ten times during the meat sample preparation, the sensitivity of the ELISAs must be at least ten times lower than the MRL. None of the three antibody ciELISAs nor antigen ciELISAs is able to detect all penicillins at 5 or 30 ng/ml. From the limit of detection concentrations (LOD, table 5.4, without hydrolysis) it is clear that the antibody ciELISA with pAb K2 is only sensitive enough for ampicillin and oxacillin (LOD = 1 and 7 ng/ml, respectively). The antibody ciELISA with pAb K8 can only detect ampicillin and benzylpenicillin at the MRL (LOD = 1.5 and 5 ng/ml, respectively) and with pAb K6 only ampicillin (LOD = 0.3 ng/ml). Because the antibody ciELISA was more sensitive for all three antisera than the antigen ciELISA, the first one was chosen for further improvement of the assay.

Physiological penicillin-protein conjugates were used to induce the polyclonal antibodies. In such conjugates, the β-lactam ring of the penicillins is open (Cliquet et al., 2001). Therefore it could be expected that the pAb have a higher affinity for the open ring structure of penicillins. Antibodies elicited by a certain conformation of the antigen are extremely sensitive to this form of the antigen (Jemmerson, 1995). The β-lactam ring can hydrolyse spontaneously when dissolving penicillins in an aqueous solution or can be

hydrolysed enzymatically with the enzyme Penicillinase I (Kleirolomoon et al., 1999, Boison et al., 1995). Even cloxacillin, considered to be poorly hydrolysed by penicillinase from *B. cereus* (Bush et al., 1995), can be hydrolysed by this enzyme (one unit for 1 µg of cloxacillin) within 30 min (Grubelnik et al., 2003; Fink et al., 1987). The structure of the native and hydrolysed ampicillin molecule is shown in Figure 5.2.

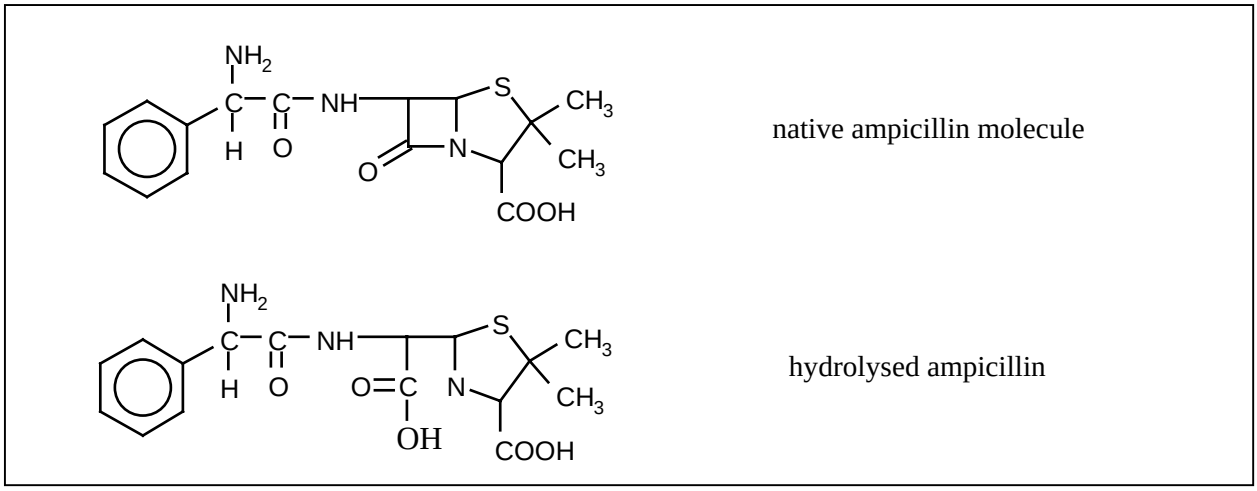


Figure 5.2: Structure of the hydrolysed and native ampicillin molecule

To investigate the influence of the hydrolysis of penicillins on the recognition by the antibodies in the antibody ciELISA, buffer dilutions of hydrolysed and non-hydrolysed penicillin were tested for each penicillin using the three pAbs (Table 5.2 and 5.4). Two methods for hydrolysis were compared: spontaneous hydrolysis by incubating a penicillin solution overnight at room temperature and enzyme mediated hydrolysis. Care was taken while preparing the samples containing non-hydrolysed penicillins to avoid spontaneous hydrolysis: the penicillins were dissolved in buffer just before the samples were added to the ELISA. From the results it is clear that the hydrolysis of the sample penicillins strongly improved the sensitivity of the assay. The enzyme-mediated hydrolysis is even more efficient than the spontaneous hydrolysis. The LOD of all tested penicillins in buffer solution analysed after enzyme hydrolysis is lower than 5 ng/ml or 30 ng/ml in each antibody ciELISA (Table 5.4), except for the detection of amoxicillin with pAb K6.

The same approach was followed by Grubelnik and coworkers (2001). They developed ELISAs for the detection of benzylpenicillin, resp. cloxacillin using enzymatic hydrolysis (with Penicillinase I) and antibodies specific for the hydrolysed form of the antibiotics. Hydrolysis of the antibiotics induced a shift of the IC50-value from 3.5 ng/ml to 0.2 ng/ml in the case of benzylpenicillin and from 2 ng/ml to 0.65 ng/ml in the case of

cloxacillin. In our ELISAs, the impact of hydrolysis was more radical. Using the pAb K2 for example, a shift of the IC50-value from 450 ng/ml to 7 ng/ml in the case of benzylpenicillin and from 800 ng/ml to 50 ng/ml in the case of cloxacillin. Grubelnik and coworkers (2001) used 20 to 100 times more Penicillinase I for hydrolysis purpose as compared to our procedure. The difference of effect of hydrolysis is probably due to the different concentration range at which the antibiotics are hydrolysed.

Thus, for the three antisera pAb K2, pAb K6 and pAb K8, the detection of all penicillins in buffer solutions below the MRL in the antibody ciELISA was achieved when the penicillins were hydrolysed with Penicillinase I. In table 5.5, a comparison is made between the antisera pAb K2 and pAb K8 and the antisera obtained by other investigators (Usleber et al., 1998; Grubelnik et al., 2001).

Table 5.5: Comparison of the IC50-values for six penicillins obtained for antisera pAb K2 and pAb K8 (hydrolysed, antibody ciELISA), and three antisera described in literature.

Antiserum	pAb K2	pAb K8	pAb Usleber[1]	pAb Grubelnik[2]	pAb Grubelnik[2]
Immunogen[3]	amp-bsa, bpg-bsa	amp-bsa, bpg-bsa, ox-bsa, diclox-bsa	amp-glut-bsa	bpg-bsa	clox-bsa
Animal species	rabbit	rabbit	rabbit	rabbit	Rabbit
	IC50-values (ng/ml)				
Ampicillin	0.08	2	42	4.6	$>10^5$
Amoxicillin	2	3.5	44	2500	$>10^5$
Benzylpenicillin	7	0.8	18	0.2	$>10^5$
Oxacillin	6	10	22	3000	29
Cloxacillin	50	10	48	140	0.65
Dicloxacillin	200	6	44	3000	105

[1]Usleber et al., 1998
[2]Grubelnik et al., 2001
[3]pen-bsa = physiologic penicillin-bovine serum albumin conjugate; amp-glut-bsa = ampicillin coupled to bovine serum albumin using glutaraldehyde as linker molecule

With the antisera pAb K2 or pAb K8 lower IC50-value, and thus higher sensitivities, were obtained for all tested penicillins as compared to the properties of the antisera described by Grubelnik and coworkers (2001), except for cloxacillin. This can be explained by the fact that we did not immunize the rabbits with a cloxacillin conjugate. The antisera of Grubelnik and coworkers (2001) are clearly more specific for the penicillin used as hapten. Thus, the broad specificity and sensitivity of the antisera pAb K2 and pAb K8 can be ascribed to the alternately administration of different physiological penicillin-protein conjugates for

immunization. With the antisera pAb K8 lower IC50-values, and thus higher sensitivities, were obtained for all tested penicillins as compared to the antiserum described by Usleber and coworkers (1998). This antiserum was obtained after immunization with a glutaraldehyde coupled ampicillin-protein conjugate and is specific for the intact β-lactam structure of penicillins. The conjugate was found to be a weak immunogen since only one of three rabbits produced a penicillin-specific antiserum. They observed no cross-reaction with hydrolysed penicillins. We demonstrated that glutaraldehyde conjugates were weaker conjugates than physiological penicillin-protein conjugates (Cliquet et al., 2001).

With the antisera of Usleber and coworkers (1998) a test system is developed, not sensitive enough and detects the intact penicillin structure. The MRL is set up for this structure. With our antisera, a sensitive method is developed, that mainly detects the degradation products, which are not taken into account in the MRL. However, the detection of the hydrolysed penicillin can be important. Although no scientific evidence is provided that people can have adverse reactions after intake of penicillin through food products, the risk in case of already sensitised individuals may not be underestimated (Dewdney, 1991). De Baere and coworkers (2002) detected high amounts of metabolites after administration of amoxycillin to pigs, contrary to what is usually assumed. Furthermore, spontaneous hydrolysis of penicillins will occur during sample handling and will always give an underestimation of the intact penicillin concentration.

Acknowledgements: This study is supported by the Ministry of Agriculture, Brussels, Belgium and by the Institute for Veterinary Inspection (IVK). The technical assistance of Els De Vogelaere was greatly appreciated.

Chapter 6

Penicillin-specific antibodies: monoclonals versus polyclonals in ELISA and in an optical biosensor

Based on: P. Cliquet, B. M. Goddeeris, K. Bonroy, and E. Cox. Penicillin-specific antibodies: monoclonals versus polyclonals in ELISA and in an optical biosensor. Food and Agricultural Immunology, 2004, accepted.

Abstract

Two penicillin-specific monoclonal antibodies mAb 19C9 and mAb 9H3 and the penicillin-specific polyclonal antibodies pAb K2 were evaluated for their use in a competitive inhibition (ci)ELISA and in the BIAcore™ optical biosensor. In the ciELISA, an ampicillin-protein conjugate was used as coating molecule. For the biosensor assay, ampicillin was immobilized on a CM5 chip. With both monoclonal antibodies and in both testsystems, ampicillin, amoxicillin and benzylpenicillin were better recognized than oxacillin, cloxacillin and dicloxacillin. Because the reproducibility was better in the biosensor (CV = 1.6 %) than in the ciELISA (CV = 8.9 %), the limit of detection for ampicillin in buffer solution using mAb 19C9 was lower in the biosensor (46 ng/ml) as compared to the ciELISA (356 ng/ml). Ampicillin can thus be detected below the MRL (50 ng/ml) in the biosensor assay but not in the ciELISA.

Both the ELISA and biosensor assay using the polyclonal antibodies pAb K2 were more sensitive as compared to the assays with the monoclonals. The ELISA using pAb K2 allowed the detection of all tested penicillins below the MRL. In the biosensor assay, ampicillin was also detected below the MRL (IC50 = 10 ng/ml). In contrast to the binding of the monoclonals, no spontaneous dissociation was observed after injection of the polyclonal antibodies in the biosensor. Whereas the monoclonals were completely removed from the sensor surface using ampicillin in buffer solution as regeneration solution, stronger conditions were necessary for the pAb binding.

Keywords: ELISA- optical biosensor- monoclonal and polyclonal antibodies-penicillins

Introduction

Penicillins are widely used for the treatment of bacterial infectious diseases. Despite their low toxicity, residues of penicillins in food can be harmful for the consumer (Milhaud & Person, 1981). Traditionally, microbiological methods and receptor assays are used to trace the presence of anti-microbial drug residues (Allison, 1985; Charm and Chi, 1988; Kavanagh, 1989). Such screening tests are usually cheap and easy to perform, but only the active form of an antibiotic is detected, not its residue. Penicillin residues have lost their anti-microbial activity but can still act as an allergen (Dewdney et al., 1991). An alternative for the

microbiological assays is the detection of residues by ELISA (Paraf and Peltre, 1991) or using a biosensor assay (Sternesjo et al., 1995).

ELISA offers the possibility of a very quick, specific and sensitive assay in addition to the advantage of analysing more samples simultaneously. Using antibodies specific for the common structure of penicillin residues, it should be possible to develop an ELISA for the detection of all penicillins in one analysis. Indeed, penicillins share a 6-aminopenicillanic acid structure: a ß-lactam ring coupled to a thiazolidin ring (Figure 2.1, Chapter 2). In a previous study, two monoclonal antibodies (mAb 19C9 and mAb 9H3) and polyclonal antibodies (pAb K2) specific for the common structure of penicillins were obtained after immunization of mice, respectively rabbits with physiological ampicillin-protein conjugates (Cliquet et al., 2001; Cliquet et al., submitted). With the polyclonal antibodies, a competitive inhibition ELISA was developed in which all penicillins can be detected at the MRL (Cliquet et al., submitted).

Optical biosensors, such as BIAcore™ are becoming widely considered for food quality and safety control. The BIAcore™ technology provides fast, automated, reliable, robust and high capacity multi-residue analysis. Analysis of one sample is completed within minutes. Immunobiosensor assays for the detection of sulfonamide residues in milk, chicken sera, porcine bile and muscle tissues have been described (Sternesjo et al., 1995; Crooks et al., 1998; Elliott et al., 1999; Bjurling et al., 2000; Haasnoot et al., 2003). Test kits for clenbuterol, streptomycine, sulfadiazine, sulfamethazine, and for the group-specific detection of all sulfonamides are already commercially available.

In the BIAcore™ biosensor, one reactant is immobilized onto the sensor surface and the other is injected over the surface using a constant flow rate (Panayotou, 1998). Because the response is proportional to the mass of bound analyte, the binding of low molecular weight analytes may be difficult to detect. In that case a competitive assay can be used: a high molecular weight molecule (residue-specific antibody) and a low molecular weight analyte (residue) are together injected over the sensor surface with the immobilized residue (Karlsson and Stahlberg, 1995). However, the BIAcore™ 3000 allows monitoring the binding of molecules with molecular weights as low as 180 dalton (Markey, 1998; Karlsson et al., 2000; Haasnoot et al., 2002). Thus, it may be possible to monitor the binding of penicillins (300 – 600 D) to immobilized penicillin-specific antibodies. Interesting about this biosensor is that interactions are measured in real time so that binding of low affinity antibodies can be

observed, whereas in ELISA low affinity bindings are washed away. Furthermore, reactants need not to be labelled, as compared to ELISA (Panayotou, 1998; Tijssen, 1985).

The aim of the present study was to develop a sensitive competitive inhibition ELISA using the monoclonal antibodies mAb 19C9 and mAb 9H3, and compare the detection of penicillins in the ELISA with the previously described ELISA using the polyclonals pAb K2. The binding properties of the antibodies were investigated in the BIAcore™ biosensor to explore the possibility to develop a biosensor assay for the detection of penicillins using our antibodies.

Material and methods

Reagents and chemicals

Benzylpenicillin, oxacillin, dicloxacillin, carbenicillin, 6-aminopenicillanic acid, cefaclor, cephadrin, cephalexin, sulfanilamide, chloramphenicol, streptomycin, 3-maleimido benzoyl-*N*-hydroxysuccinimide ester (MBS), Penicillinase (EC 3.5.2.6, type 1, from *B. cereus*), bovine serum albumin (BSA), ovalbumin (OVA) and 3, 3', 5, 5'-tetramethylbenzidin (TMB) were purchased from Sigma Chemicals (Bornem, Belgium). The TMB enzyme-substrate solution was prepared by adding 100 µl of a TMB stock solution (10 mg TMB dissolved in 1 ml dimethylsulfoxide) to 10 ml phosphate-citrate buffer pH 5 (25.7 ml 0.2 M Na_2HPO_4 + 24.3 ml 0.1 M citrate + 50 ml distilled water) supplemented with 1.3 µl H_2O_2. Amoxicillin and cloxacillin were from ICN Biochemicals (Asse-Relegem, Belgium) and ampicillin and ABTS® (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Tablet and buffer) from Roche Diagnostics (Brussels, Belgium). The ABTS enzyme-substrate solution was prepared by dissolving 1 ABTS tablet® (50 mg) in 50 ml ABTS buffer®. Tween® 20 (polyoxyethylene sorbitan monolaurate) and neomycin were purchased from Merck-Belgolabo (Overijse, Belgium). EZ-link™ Sulfo-NHS-LC-Biotin was purchased from Pierce (Perbio, Erembodegem-Aalst, Belgium). Peroxidase-labelled rabbit anti-mouse immunoglobulins (code n°P0260) and peroxidase-labelled swine anti-rabbit immunoglobulins (code n° P0217) were from DAKO Diagnostica (Prosan, Ghent, Belgium). ELISA microtiter plates (Maxisorp®) were provided by NUNC (VWR international, Leuven, Belgium). All other chemicals were of reagent grade.

The CM5 sensor chips, amine coupling kit (*N*-hydroxisuccinimide (NHS), *N*-ethyl-*N*'-(3-diethylaminopropyl) carbodiimide (EDC) and ethanolamine-HCl) and 10mM hepes buffered saline pH 7.4 (HBS) were purchased from BIAcore (Uppsala, Sweden).

The ELISA coating antigen, an ampicillin-ovalbumin conjugate (ampMBSova), was synthesized with an activated ester method using the hetero-bifunctional reagent *m*-maleimido benzoyl-*N*-hydroxisuccinimide ester (MBS) (Cliquet et al., 2001).

Penicillin-specific antibodies

Two monoclonal antibodies (mAb 19C9 and mAb 9H3; ascites fluids treated with saturated ammonium sulfate) and the polyclonal antiserum pAb K2 (sera treated with kaolin, Van den Broeck et al., 1999) were obtained after immunization of Balb/c mice, respectively rabbits with ampicillin and benzylpenicillin coupled without cross-linker to BSA (Cliquet et al., 2001; Cliquet et al., submitted).

Biotinylation of ampicillin

Ampicillin (1.2 mg) was diluted in 300 µl phosphate buffer 0.1 M, pH 7.2 and added to 3.7 mg of a succinimide ester-biotin conjugate (EZ-link™ Sulfo-NHS-LC-Biotin) diluted in 700 µl phosphate buffer 0.1 M, pH 7.2. The reaction mixture was incubated during 24 hours at room temperature while shaking. Aliquots of the conjugate were stored at –20 °C.

Hydrolysis of penicillins

Penicillinase was diluted in PBS (1000 unit/ml) and stored at –80°C. Prior to use, the penicillinase I stock was further diluted (0.1 unit/ml). Five microliter of this penicillinase dilution was added to 1 ml sample. Next, the mixture was incubated during one hour at 37°C followed by a 30 min incubation at 4°C to inactivate the enzyme (Medina et al., 1998).

Competitive inhibition ELISA coated with antigen (antigen ciELISA)

Microtiter plates were coated overnight with 2.5 µg/ml ampMBSova diluted in bicarbonate coating buffer (0.05 M; pH 9.4) followed by blocking for two hours at 37 °C with 5% glycine in coating buffer. Subsequently, the samples were added diluted in PBS (0.15 M; pH 7.4) containing 3% BSA and 0.05% Tween®20 (dilution buffer) together with an appropriate concentration of the penicillin-specific antibodies (mAb 19C9: 0.46 µg/ml; mAb 9H3: 0.53 µg/ml, pAb K2: 0.27 µg/ml). The microtiter plates were then incubated for one hour at 37 °C with mAb 9H3 or pAb K2, and for 30 min at 4 °C with mAb 19C9. Next, the

plates were incubated with the secondary antibodies in dilution buffer for 60 min at 37 °C. Between each step, the plates were washed three times with PBS containing 0.05 % Tween®20. Finally, the plates were incubated for one hour at 37 °C with the enzyme substrate solution (ABTS, 50 µl/well). The colour development (absorbance (A) or optical density (OD)) was measured at 405 nm using an ELISA reader (Spectrofluor, TECAN). The competition in the ELISA between a free penicillin in the sample and the coated ampicillin (ampMBSova) is calculated with the formula: competition (%) = (1- (A/A_o))*100 with A = absorbance of a tested sample solution and A_o the absorbance of the solution without penicillin.

Competitive inhibition (ci)ELISA coated with antibody (antibody ciELISA)

Microtiter plates were coated overnight with pAb K2 (2 µg/ml) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). After coating, the plates were blocked for two hours at 37 °C with 0.2 % Tween®80 in the coating buffer. Subsequently, the samples were added (100 µl/well) in dilution buffer, as for the antigen ciELISA. The plates were then incubated for one hour at 4 °C. Next, 100 µl of biotinylated ampicillin in dilution buffer was added, without washing the plate, for 30 min at 4 °C. The other wash steps occurred as for the antigen ciELISA. Then, the plates were incubated for 30 min at 37 °C with streptavidin-peroxidase conjugate in dilution buffer. Finally, the enzyme substrate solution (TMB) was added to the plates for one hour at 37 °C. The colour development was measured at 650 nm. The competition in the ELISA between a free penicillin in the sample and the biotinylated ampicillin is calculated with the same formula as in the antigen ciELISA: competition (%) = (1- (A/A_o))*100 with A = absorbance of a tested sample solution and A_o the absorbance of a similar solution without a penicillin.

Biosensor

Instrumentation: The optical biosensor BIAcore™ 3000 system from BIAcore (Uppsala, Sweden) was used. Data analysis was performed using BIAevaluation 3.1 software.

Sensor surface:

1) Immobilization of penicillin: ampicillin was covalently immobilized to the surface of one of the four channels of the sensor chip by subsequent injections of several reagents via the autoinjector unit of the BIAcore™ instrument. All injections were performed at a flow rate of 10 µl/min. First, the carboxyl groups of the dextran layer of the sensor chip were activated

by injection of 70 µl of a mixture of EDC and NHS (1:1). Then, 70 µl of 10 mM ampicillin diluted in borate buffer 50 mM pH 8.5 was injected. To block the remaining reactive carboxyl groups of the dextran layer 10 µl of 1 M ethanolamine-HCl was injected. Another channel was designed as reference channel and was just activated and blocked.

2) Immobilization of antibody: mAb 19C9 was covalently bound using the immobilization wizard of the BIAcore™ 3000 control 3.1.1 software. After activation of the carboxyl groups of the dextran layer, mAb 19C9 (100 µg/ml diluted in 5 mM maleate buffer pH 6) was injected until a signal of 25 000 RU (Response Units) was achieved. The remaining reactive carboxyl groups of the dextran layer were blocked with an injection of 10 µl of 1 M ethanolamine-HCl. Another channel was just activated and blocked for use as reference channel.

Sample analysis on immobilized ampicillin:

The method was designed as an inhibition assay. A fixed concentration of antibody is mixed with the sample prior to injection. Binding of the penicillin-specific antibodies on the prepared surface is measured. Any penicillin in the sample will bind to the antibody and subsequently inhibit the antibody binding to the surface. The response is inversely related to the amount of penicillin present in the sample. The surface is then regenerated, ready for the next sample.

When the samples were mixed with an equal volume of mAb (22 µg/ml for mAb 19C9, 96 µg/ml for mAb 9H3, in PBS), the mixtures were injected during 7 min with a constant flow rate of 40 µl/min. Regeneration of the sensor surface was achieved with an excess of ampicillin (20 µl of 500 µg/ml ampicillin). Each cycle took about 10 min. Between two analyses the sensor surface was rinsed with HBS.

When the samples were mixed with an equal volume of pAb K2 (50 µg/ml), they were injected during 4 min with a flow rate of 20 µl/min. For regeneration, different solutions were tried out: excess of ampicillin (500 µg/ml), sodium acetate (10 mM), sodium hydroxide (50 mM), glycine solution (pH3), sodium hydroxide (0.1 M) containing 20% acetonitril.

Sample analysis on immobilized mAb 19C9

A constant flow rate of 10 µl/min was used during the analysis. Samples consisted of a known concentration of penicillin or ampMBSova in PBS. The samples were injected during 4 min. For regeneration, different solutions were tried out: excess of ampicillin (500 µg/ml), sodium acetate (10 mM), sodium hydroxide (50 mM), glycine solution (pH 3). Between two analyses the sensor chip was rinsed with HBS.

Storage: Between each analysis, the chip was rinsed with HBS at a flow rate of 10 µl/min. Otherwise, the chips were removed and stored dry at 4 °C.

Results and discussion

Development of an ELISA with the monoclonal antibodies

The IC50-values (the concentration at which the binding of the antibody to the coating was inhibited for 50%) for different penicillins in buffer solution analysed in the antigen ciELISA with mAb 19C9, demonstrate that the ELISA was most sensitive for amoxicillin, followed by ampicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin (Table 6.1). The ELISA with mAb 9H3 was slightly less sensitive for ampicillin, amoxicillin, oxacillin, cloxacillin and dicloxacillin than the ELISA with mAb 19C9. However, both monoclonals recognized ampicillin, amoxicillin and benzylpenicillin better than oxacillin, cloxacillin and dicloxacillin. The latter ones have a larger side chain (Figure 2.1, Chapter 2), possibly causing steric hindrance for the binding of the monoclonals to the common penicillin core.

Table 6.1: Concentration at 50% competition (IC50, ng/ml) and limit of detection (LOD, ng/ml) for different penicillins detected with mAb 19C9 and mAb 9H3 in the antigen ciELISA.

Penicillin	IC50 (ng/ml)		LOD (ng/ml)	
	mAb 19C9	mAb 9H3	mAb 19C9	mAb 9H3
Ampicillin	1350	3500	356	782
Benzylpenicillin	3100	1930	729	325
Amoxicillin	140	1650	18	257
Oxacillin	5580	18040	1029	2660
Cloxacillin	9850	16270	2599	2431
Dicloxacillin	15500	20050	3702	3598

The monoclonals are highly specific for penicillins. No cross-reactions were observed for cephalosporins (cephadrin, cephalexin, cefaclor), clavulanic acid, sulfanilamide, chloramphenicol, neomycin and streptomycin analysed in the ELISA at a concentration of 20 µg/ml. Low cross-reactivities were noticed for carbenicillin and 6-aminopenicillanic acid (IC50 > 20 µg/ml). The monoclonals were deduced from mice immunized with physiological penicillin-protein conjugates (Cliquet et al., 2001). In such conjugates, the β-lactam ring of the penicillins is open. As a result, antibodies elicited with these conjugates could have higher affinity for hydrolysed penicillins as compared to the native molecule. However, mAb 19C9

and mAb 9H3 showed lower recognition for some penicillins (ampicillin, amoxicillin, benzylpenicillin and oxacillin for mAb 19C9; ampicillin, amoxicillin for mAb 9H3) treated with Penicillinase as compared to these penicillins freshly dissolved in buffer before analyses and thus not hydrolysed. In resume, both the β-lactam ring and the thiazolidin ring are important for the detection by the monoclonals since cephalosporins are not recognized and some hydrolysed penicillins are less well recognized than the native penicillins.

No ciELISA coated with antibodies (antibody ciELISA) could be developed using the monoclonal antibodies. Different strategies for immobilization of the monoclonals to the assay surface were tried out: passive coating on MAXIsorp and POLYsorp plates, immobilization on precoated surface with mouse-specific antibodies or with protein G, and covalent binding of the monoclonals to COVALINK-aminoplates (Nunc), CARBObind plates (Costar), and SULFHYDRYLbind plates (Costar). Immobilization of the monoclonals was first controlled using enzyme labelled mouse-specific antibodies and next with biotinylated ampicillin. In all cases, immobilized monoclonal antibodies were detected. However, specific binding of the biotinylated ampicillin to the immobilized antibodies could never be achieved.

Monoclonals vs polyclonal antibodies in ELISA

In contrast to using the monoclonals, an antibody ciELISA could be developed using the polyclonal antibodies pAb K2. Moreover, with the pAb K2, a better recognition of all tested penicillins was observed in the antibody ciELISA as compared to the antigen ciELISA. The sensitivity of the ELISA using the polyclonals was improved when the penicillin samples were first hydrolysed with Penicillinase (Table 6.2; Cliquet et al., submitted).

The MRL-value for ampicillin, benzylpenicillin and amoxicillin is 50 ppb (µg/kg, ng/ml) in meat products and 4 ppb in milk and for oxacillin, cloxacillin and dicloxacillin 300 ppb in meat and 30 ppb in milk (Anonymous, 1990). From the LOD-values (limit of detection, table 6.2) it is clear that all penicillins can be detected at their MRL in the antibody ciELISA using the pAb K2. The MRL is not reached for any penicillin when mAb 9H3 is used (LOD > 50 ng/ml, table 6.1). With mAb 19C9, only amoxicillin (LOD = 18 ng/ml) is detected at the MRL (Table 6.1). However, when analysing meat samples in ELISA, the penicillins have to be extracted from the meat. During this step, the samples become diluted ten times so that the ELISA must be at least ten times more sensitive than the MRL-value. Different attempts were made to improve the sensitivity of the antigen ciELISA using mAb

19C9: e.g. different buffer solutions (pH, salts), different incubation temperature, polyethylene glycol addition to the sample incubation step, different enzyme labelled conjugates, … (Cliquet et al., 1998). None of them did increase the sensitivity significantly, except for lowering the incubation temperature. The sensitivity of the detection of free penicillins by mAb 19C9 was improved when the assay was performed at 4 °C instead of 37°C or room temperature (RT).

Table 6.2: Concentration at 50% competition (IC50, ng/ml) and limit of detection (LOD, ng/ml) for different penicillins (hydrolysed or not) detected with pAb K2 in the antigen ciELISA (Ag coat) and in the antibody ciELISA (Ab coat).

Penicillin	IC50 (ng/ml)			LOD (ng/ml)		
	Ag coat	Ab coat		Ag coat	Ab coat	
	not hydrolysed	not hydrolysed	hydrolysed	not hydrolysed	not hydrolysed	hydrolysed
Ampicillin	1000	50	0.08	15	1	<0.1
Benzylpenicillin	1800	450	7	150	30	1
Amoxicillin	6000	150	2	200	10	<1
Oxacillin	1000	150	6	500	7	0.6
Cloxacillin	8000	800	50	550	60	7
Dicloxacillin	20000	> 1000	200	1050	200	30

Detection of penicillins in the biosensor assay with immobilized antibodies

Binding of low molecular weight analytes (>180 dalton) to immobilized ligand can be detected with the BIAcore™ 3000 (Markey, 1998). Haasnoot and coworkers developed such biosensor assays for gentamycin (2001) and for streptomycin (2002). Thus, it might be possible to monitor the binding of penicillins (300 – 600 D) to immobilized penicillin-specific antibodies. However, no signal was detected when ampicillin was injected over the immobilized mAb 19C9. On the other hand, a strong interaction between ampMBSova (the coating antigen of the ciELISA) and the immobilized mAb 19C9 was observed (Figure 6.1), indicating a good immobilization of the mAb on the sensor surface. It is possible that the signal from the binding of ampicillin to the immobilized mAb 19C9 is masked by the background signal of the buffer (Karlsson & Falt, 1997). Indeed, because the signal is proportional to the molecular weight of the binder molecule, a small molecule will always provide a low signal, typically in the range of 5 to 100 RU. The signal can therefore be masked by the buffer bulk effect. The buffer bulk effect occurs when the sample buffer (in this case PBS) is different from the running buffer (HBS). When the same buffer composition

is used for the running buffer and the sample, it should be possible to recover the specific signal and thus make the binding events clearly visible (Karlsson & Falt, 1997). Unfortunately, this was not tested in the present study. Using a reference channel in which an irrelevant monoclonal is immobilized should also help to make the binding more visible (Haasnoot et al., 2002). In our study the reference channel was activated and deactivated in the same way as for the specific channel, but without the immobilized molecule.

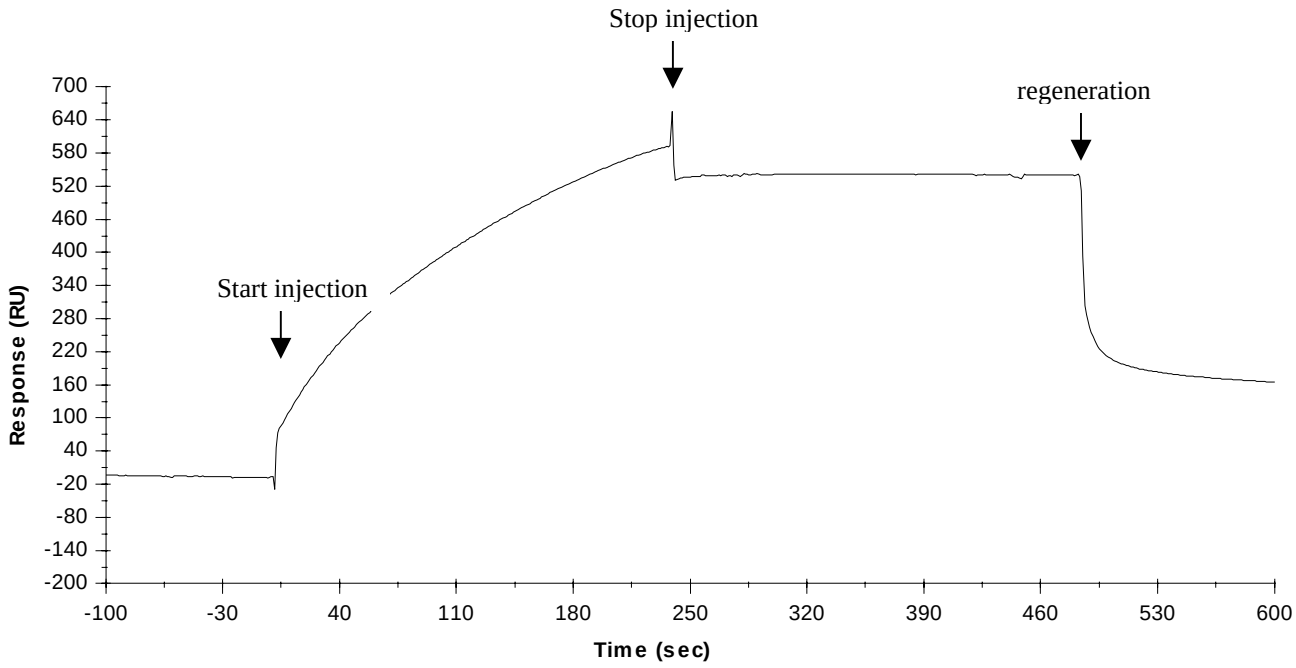


Figure 6.1: Binding of ampMBSova to the immobilized monoclonal antibodies mAb 19C9 in the biosensor.

Detection of penicillins in the biosensor assay with immobilized ampicillin

Some characteristics of the binding of mAb 19C9 and mAb 9H3 to the coated ampicillin in the ELISA and in the biosensor assay are given in table 6.3. The binding response of the monoclonals was determined by calculating the average of six runs. The variation on the average (standard deviation, SD) is much higher in the ELISA than in the biosensor assay. As a result, the reproducibility of analyses will be better in the biosensor. The limit of detection (LOD = average response – 3x SD) and the concentration at which the binding of the antibody to the coating was inhibited for 50% (expressed as IC50 value) were determined for the dose-response curve using ampicillin as free penicillin. Because the standard deviation (SD) is higher in the ELISA than in the biosensor, the LOD is lower in the

biosensor than in the ELISA. The LOD for ampicillin in buffer solution in de biosensor is 46 ng/ml. Consequently, the MRL for ampicillin is reached (50 ppb or ng/ml).

Table 6.3: Determination of the limit of detection (LOD) and concentration at 50% competition (IC50) for ampicillin detected with mAb 19C9 and mAb 9H3 in the ciELISA coated with antigen and in the biosensor, at room temperature (RT) or 4°C.

	mAb 19C9			mAb 9H3	
	ELISA 4°C	Biosensor RT	Biosensor 4°C	ELISA RT	Biosensor RT
Signal[a]	0.5167	7350	7380	0.7122	7676
SD[b]	0.0462	37	120	0.275	109.5
CV[c] (%)	8.9	0.5	1.6	3.8	1.4
LOD[d] (µg/ml)	0.356	0.054	0.046	0.782	0.030
IC50[d] (µg/ml)	1.350	>1.000	0.524	3500	>1.000

[a]Signal: average of repeated measurements of the binding of the antibody to the coated (ELISA) or immobilized (biosensor) ampicillin, in absence of penicillin in the sample. The signal measured in ELISA is expressed as absorbance (OD) and in the biosensor as Response Units (RU).
[b]SD = standard deviation of the average response or absorbance
[c]CV = coefficient of variation of the average response or absorbance
[d]LOD (= limit of detection = average signal – 3* SD) and IC50 (signal obtained when the binding of the antibodies is inhibited for 50 %): concentration (µg/ml) is obtained by extrapolation of the response or absorbance in a calibration curve. The calibration curve was established by plotting the concentration of a standard dilution of ampicillin against the signal (RU or OD) obtained for the binding of the antibodies in presence of the standard dilutions.

In ELISA the sensitivity of the detection of free penicillins by mAb 19C9 was improved when the assay was performed at 4 °C instead of 37°C or room temperature (RT) (Cliquet et al., 1998). The same observation was made in the biosensor assay (Table 6.3). The IC50 for ampicillin in the biosensor assay was lower when the runs were done at 4 °C instead of RT (0.524 and >1.000 µg/ml, respectively). The LOD however, was only slightly lower at 4°C as compared to RT (0.046 and 0.054 µg/ml, respectively). In figure 6.2 the sensorgrams are given for mAb 19C9 (4 °C *vs* RT) and mAb 9H3 (only RT). The dissociation curves reveal that the binding of mAb 19C9 to the immobilized ampicillin is stronger at 4 °C than at RT, and is stronger than the binding of mAb 9H3 (Gunnarsson, 1998).

The cross-reactivity values (CR) of several penicillins at 1 µg/ml in PBS in regard to ampicillin (CR = 100 %) were determined in the ELISA and in the biosensor assay (Table 6.4). With both antibodies, but especially with mAb 9H3, oxacillin, cloxacillin and dicloxacillin were better recognized in the biosensor system than in the ELISA. In ELISA, it was not possible to detect these penicillins at concentrations below 1 µg/ml. A possible explanation is that in ELISA the binding of the antibodies to the coating molecule is favoured above the binding to antigens with lower affinity (oxacillin, cloxacillin and dicloxacillin).

Indeed, the incubation time allows the dissociation of weak antibody-antigen interactions in the advantage of the strong antibody-antigen interactions (coating antigen), resulting in lower cross-reactivities for the lower affinity antigens (Tijssen, 1985). In the biosensor the antigen-antibody interaction is measured in real-time and therefore higher cross-reactivities can be monitored for low affinity binders.

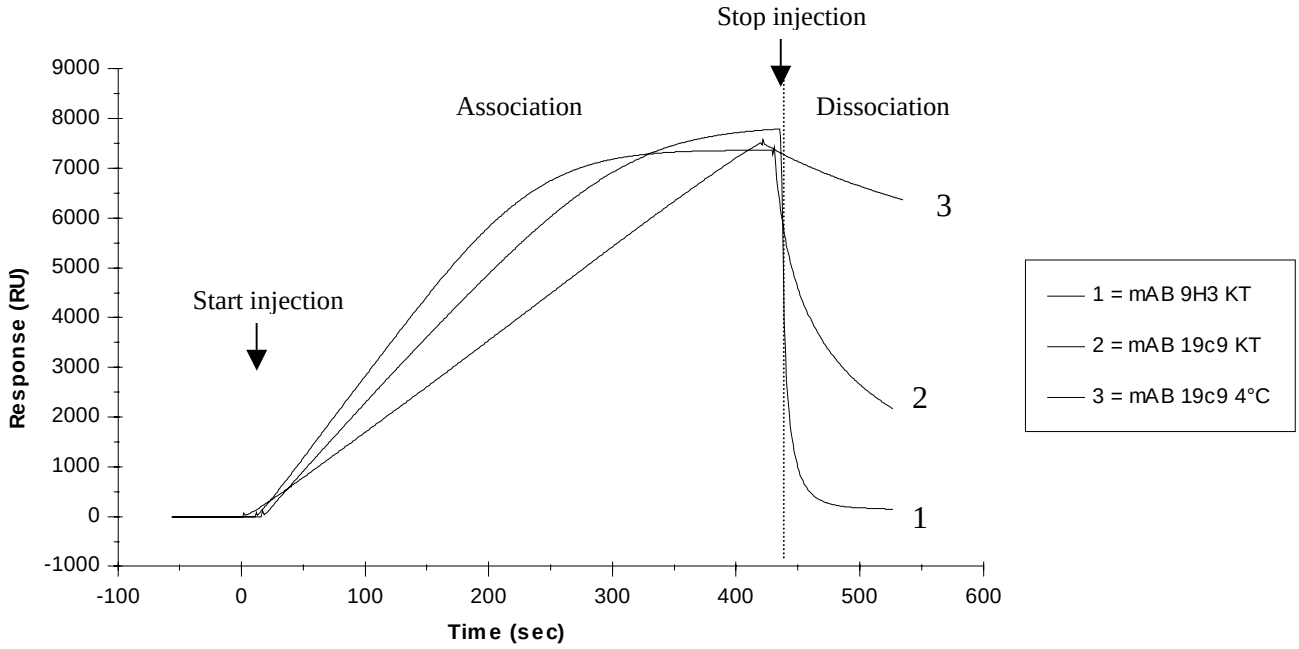


Figure 6.2: Sensorgram of the binding of mAb 19C9 (at RT and at 4°C) and of mAb 9H3 (at RT) on immobilized ampicillin in the biosensor.

Table 6.4: Cross-reactivities (%) for different penicillins (1 µg/ml) compared to ampicillin, in the antigen ciELISA and in the biosensor, using mAb 19C9 (test performed at 4°C) and using mAb 9H3 (test performed at RT).

	mAb 19C9		mAb 9H3	
Penicillin	ELISA	Biosensor	ELISA	Biosensor
Ampicillin	100	100	100	100
Amoxicillin	153	204	193	163
Benzylpenicillin	69	70	155	136
Oxacillin	-[a]	81	-	73
Cloxacillin	-	55	-	65
Dicloxacillin	-	53	-	61

[a] Cross-reactivity could not be determined because the LOD for the given penicillin is more than 1 µg/ml.

The binding of the pAb K2 on immobilized ampicillin is visualized in figure 6.3. No spontaneous dissociation of the interaction was observed what points out the high affinity of pAb K2 for ampicillin (Gunnarsson, 1998). High affinity antibodies are suitable for ELISA because the affinity of the antibody strongly influences the stability of antigen-antibody complex during the washing steps of the assay (Tijssen, 1985). Indeed, the ELISA using the pAb K2 is more sensitive for the detection of penicillins than the ELISA with the mAb (Table 6.1 and 6.2).

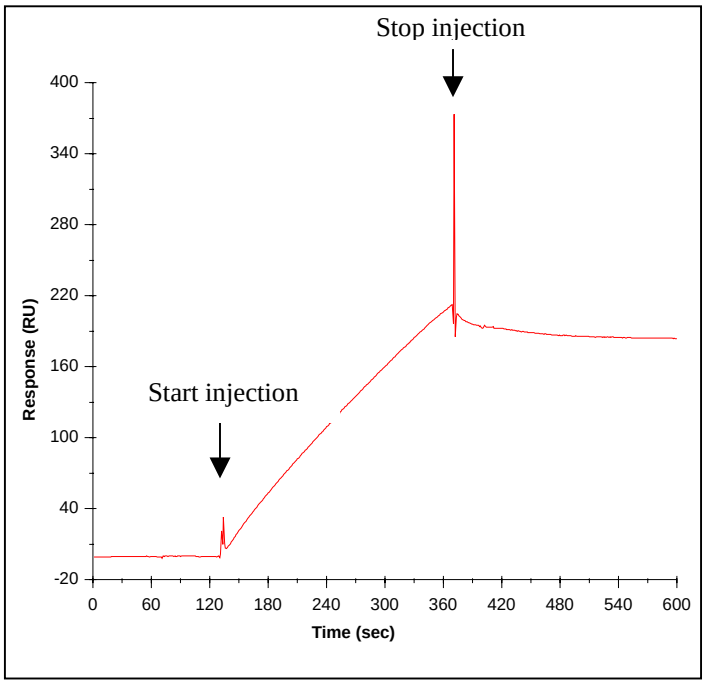


Figure 6.3: Sensorgram of the binding of pAb K2 to the immobilized ampicillin in the biosensor.

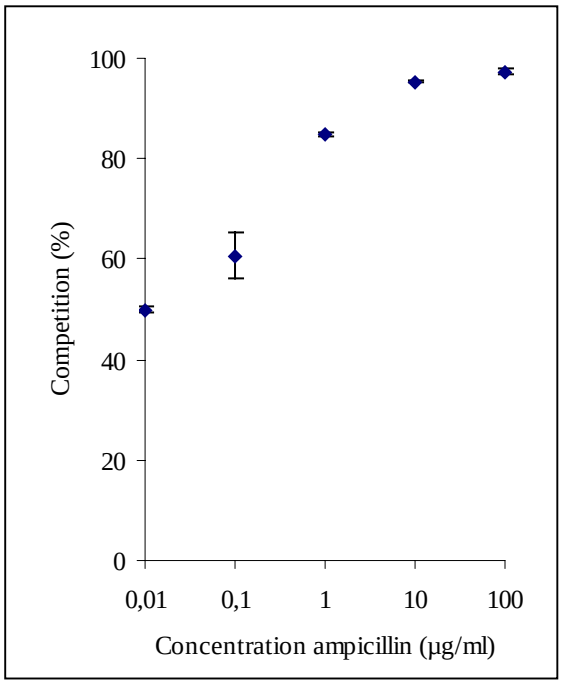


Figure 6.4: Dose-response curve for ampicillin in buffer solution analysed in the biosensor assay on immobilized ampicillin using pAb K2.

Whereas the monoclonals were completely removed from the sensor surface using ampicillin in buffer solution as regeneration solution, stronger conditions were necessary for the pAb binding. Different solutions were tried out: sodium acetate (10 mM), sodium hydroxide (50 mM), glycine solution (pH3) and sodium hydroxide (0.1 M) containing 20% acetonitril. The latter one was the most suitable one.

When the polyclonals were injected at concentrations higher than 50 µg/ml, the sensor surface could not be completely regenerated. Lower concentrations of pAb K2 led to very low specific signals (RU = 160 for 25 µg/ml). The binding of pAb K2 (50 µg/ml) to immobilized ampicillin was inhibited for 50 % in presence of 10 ng/ml ampicillin (Figure 6.4). The IC50-

value for ampicillin in the antibody ciELISA with pAb K2 is 50 ng/ml for the native molecule and 0.08 ng/ml for hydrolysed ampicillin (Table 6.3). In the biosensor assay but not in the ELISA, antibodies and ampicillin solution were preincubated. It is possible that the ampicillin solution is partially hydrolysed during this incubation. As for the ELISA, the sensitivity of the biosensor assay can probably be improved using ampicillin solutions treated with penicillinase.

Results from the biosensor assays support our findings in ELISA: the difference in sensitivity between the polyclonals and the monoclonals in ELISA can be ascribed to the fact the polyclonals have higher affinity for penicillins than the monoclonals, as was observed in the biosensor assay. Also, the improvement of the detection of penicillin in the ELISA with the monoclonals when incubated at 4°C instead of 37°C or room temperature was visualized in the biosensor. Thus, the biosensor assay helped understanding the interactions in ELISA. Using mAb 19C9, ampicillin can be detected at the MRL-value in the biosensor but not in ELISA. Moreover, using the monoclonals, the isoxazoyl penicillins are better recognized in the biosensor assay as compared to the ELISA. Thus, it should be interesting to apply the mAb for the development of a biosensor assay.

However, the results obtained in our biosensor assays should be questioned. Firstly, very high amounts of antibodies were used in the biosensor. As compared to the amounts used in ELISA, 50 times more mAb 19C9 were used, 180 times more mAb 9H3 and 185 times more pAb K2. It was expected that the biosensor assays require higher amounts of antibodies, due to the continuous flow over the sensor surface (Bergstrom, 1996). Haasnoot and coworkers (2003) reported the use of ten times more antibodies in a biosensor assay as compared to an ELISA using the same antibodies. Compared to these results, the amount of monoclonal antibodies used in our biosensor assay is tremendously high. Moreover, despite the use of such large amounts of mAb 19C9 the saturation of the immobilized surface was even only reached after 5 min at room temperature, and not reached at all after 7 min at 4°C (Figure 6.2). A possible explanation for the high amounts of antibodies is the presence of contaminating ampicillin molecules in the system, as a result of the immobilization and regeneration procedure (Bergstrom, 1996; Haasnoot et al., 2002). In both cases a large amount of ampicillin was injected into the system (259 µg for the immobilization and 10 µg for each regeneration cycle). These contaminating ampicillins can bind the antibodies so that large amounts are necessary to saturate the surface. For the mAb 19C9, this effect was more

explicit at 4°C as compared to room temperature (Figure 6.2; association curve), what seems to correspond with the higher affinity of the antibody for the free ampicillin at reduced temperature. It is recommended for low molecular weight analytes to immobilize these analytes on the sensor surface outside the system (Bergstrom, 1996, Haasnoot et al., 2003). However, other similar investigations were published where the immobilization was performed inside the system, without mentioning the risk or interference of contamination. For example, Gaudin and Marris (2000) used the same procedure as we did for the immobilization of ampicillin to a CM5 chip. Ahmad et al. (2002) even injected 3.45 mg sulfamethazine during their immobilization procedure. The authors did not mention the relative response nor the time of interaction for the antibodies. In addition the immobilization of analyte inside the system, we regenerated the surface with an excess of ampicillin to avoid the degeneration of the immobilized ampicillin when using alkali or acid regeneration conditions.

Possibly, ampicillin molecules remained in the system after the regeneration step so that for each antibody binding event a large amount of antibody was needed. Probably there was no accumulation of contaminating molecules after the regeneration step because the reproducibility of the signal of the antibody binding was very high (Coefficient of variation of six subsequent runs = 0.5%, Table 6.3).

An alternative to avoid contaminating analyte and reduce the amount of antibody is the immobilization of ampicillin-protein conjugates instead of ampicillin. Haasnoot and coworkers (2003) immobilized a sulfonamide-protein conjugate. Lower amounts of sulfonamides are available for binding to antibodies than when sulfonamides are immobilized directly on the surface like our biosensor set up. This means that we could possibly reduce the amount of antibodies by working with a surface coated with ampicillin-protein conjugates instead of ampicillin. This was indeed observed by Bonroy and coworkers (2002). In an optimised biosensor assay on an immobilized ampicillin-protein conjugate, they used less than 10 µg/ml purified mAb 19C9. They obtained better detection limits for test penicillins when analysed on a surface coated with ampicillin-protein conjugates as compared to ampicillin coating.

Thus, our biosensor assay could still be optimized. Performing the immobilization outside the system should avoid contamination with the analyte. Regeneration with an excess

of analyte should also be avoided. Moreover, immobilization of an ampicillin-protein conjugate will improve the assay sensitivity. Such immobilization could allow other regeneration conditions to be used. Nowadays, the development of a biosensor assay using mAb 19C9 is under investigation at the Interuniversity Microelectronics Center (IMEC), Heverlee, Belgium.

Conclusion

With the pAb K2, but not with the mAb 19C9 and mAb 9H3, an ELISA and a biosensor assay was developed able to detect penicillins and ampicillin, respectively, far below the MRL-value for meat. Despite the fact that the biosensor assay was not performed in optimized conditions, the monoclonals showed higher sensitivities for the penicillins in the biosensor assay as compared to the ELISA. Therefore, it should be interesting to apply the monoclonals for the development of a suitable biosensor assay.

Acknowledgements: This study is supported by the Ministry of Agriculture, Brussels, Belgium and bij the Institute of Veterinary Inspection (IVK). Special thanks to Ron Wolbert from BIAcore for the use of the BIAcore™ 3000 biosensor. The technical assistance of Els De Vogelaere was greatly appreciated.

Chapter 7

Detection of penicillins in tissue samples using the ciELISA with polyclonal antibodies pAb K2

Adapted from: *P. Cliquet, E. Cox, L. Okerman, K. De Wasch, B. M. Goddeeris. ELISA for the detection of residues of the penicillin group in food of animal origin. Med. Fac. Landbouww. Univ. Gent. 1999, 64(5b) 507-512.*

Abstract

Extraction procedures were developed for the analysis of porcine and bovine tissues in two different competitive inhibition ELISAs using the same penicillin-specific polyclonal rabbit antibodies (pAb K2): an antigen ciELISA in which antigen was coated on the plate and an antibody ciELISA coated with the polyclonal antibodies. A rather simple extraction procedure was developed for the detection of ampicillin in porcine kidney, muscle and liver tissues in the antigen ciELISA with pAb K2, using two phosphate buffer solutions (Sodium phosphate pH 5 and phosphate buffered saline pH 7.4, respectively), a centrifugation step, a filtration step and a pH adjustment. For the analysis of penicillins in incurred samples in the antibody ciELISA with pAb K2, tissue fluids were used instead of the whole meat matrix, what simplifies the sample preparation. Before analysis in ELISA, the fluids were treated with kaolin to reduce the background signals in ELISA.

The specificity, sensitivity, repeatability, decision limit (CCα) and detection capability (CCβ) were determined for both assays. The antibody ciELISA using pAb K2 is preferred above the antigen ciELISA with pAb K2 because the detection limit of the antibody ciELISA for penicillins is far below the MRL. Furthermore, the precision of the antibody ciELISA is higher than the precision of the antigen ciELISA: the range between CCα and CCβ is smaller for the analysis of kidney samples in the antibody ciELISA (9 – 19 ng/ml) as compared to the antigen ciELISA (21-69 ng/ml).

The suitability of the antibody ciELISA and the respectively extraction procedure for screening purposes was demonstrated by comparison of the analysis of incurred samples using different screening assays. Almost 100 % correlation (r = 0.96) was found between the ELISA and a commercial immunochemical method, the Parallux™ assay.

Introduction

High specific monoclonal and polyclonal antibodies were induced against the thiazolidin ring of the common 6-aminopenicillanic acid core of the β-lactam antibiotics after immunization of respectively mice and rabbits with ampicillin- and benzylpenicillin-carrier conjugates (Cliquet et al, 2001; Cliquet et al., submitted). With the monoclonals (mAb 19C9 and mAb 9H3) as well as with the polyclonal antibodies (pAb K2, pAb K6 and pAb K8), an antigen ciELISA was developed for the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin in buffer solutions (Cliquet et al., submitted; Cliquet et

al., 2004). However, the antigen ciELISAs were not able to detect all tested penicillins at the MRL (50 µg/kg for ampicillin, amoxicillin and benzylpenicillin; 300 µg/kg for oxacillin, cloxacillin and dicloxacillin). Only ampicillin could be detected below the MRL in the antigen ciELISA with the polyclonals (LOD = 6 µg/kg with pAb K2; LOD = 20 µg/ml with pAb K6 and pAb K8) and amoxicillin in the antigen ciELISA with mAb 19C9 (LOD = 18 µg/kg). With the polyclonals, but not with the monoclonals, an antibody ciELISA could be developed that was ten times more sensitive than the antigen ciELISA (Cliquet et al., submitted). Therefore only the ELISA using pAb K2 was chosen to assess the possibility of analysing porcine or bovine tissues according to the requirements for screening tests used for the inspection of food derived from treated animals (Anonymous, 2002).

Each member of the European Union has a monitoring program to test for the presence of veterinary drugs in edible tissues, according to Council Directive 96/23/EC. In Belgium, the residue control program for meat and meat products is currently operating in three steps. First, the kidney and sometimes muscle tissues collected at slaughterhouse are analysed using the New Belgian Kidney Test (NBKT) for the presence of antibiotics and other anti-microbiological substances (pre-screening). The NBKT is a microbiological assay based on the growth inhibition of bacteria on an agar plate in the presence of a paper disk impregnated with kidney fluid or a piece of tissue (Okerman, 1995). The assay only indicates the presence of an anti-microbial agent but does not discriminate between different families of antibiotics or chemotherapeutics. Therefore, positive samples are further screened using other microbiological assays or using immunoassays to identify the inhibitory substance or the group to which it belongs (screening). Finally, the present analyte is identified and quantified using a confirmatory method (confirmation).

Any method or combination of methods may only be used for screening or confirmatory purposes if it can be proven that they fulfill the relevant requirements established in the Commission Decision 2002/657/EC. For screening purpose, only those methods are allowed that are validated and have a false-compliant rate lower than 5% at the MRL. Validation of a method means the demonstration that the method complies with the criteria applicable for the relevant performance characteristics. For a qualitative screening method, the detection capability (CCβ), the selectivity/specificity and the

applicability/ruggedness/stability must be demonstrated. When a screening assay will be used quantitatively, the precision must additionally be determined.

The aim of this study was firstly to develop an appropriate extraction procedure for the detection of penicillins in porcine tissues in the antigen ciELISA and in the antibody ciELISA using pAb K2. In a second step, it was investigated if the ELISAs could be validated according to the Commission Decision 2002/657/EC. Some of the relevant performance characteristics for the validation of both assays were determined and are discussed in this study. Thirdly, the analysis of incurred samples using the ELISA procedures was compared to the analysis of the same samples using a confirmation method or other screening tests.

Material and methods

Origin of the animal tissue samples

Twelve pigs (22kg-43kg) were injected twice intramuscularly in the neck with an interval of 12 hours with ampicillin trihydrate (AMPILUX 200, Pharmalux Belgium, withdrawal time = 6 days). The doses used as well as the interval between the second injection and the time of slaughter, are presented in Tables 7.4, 7.5 and 7.6. The injections were performed in such a way that all pigs could be slaughtered at the same day. Euthanasia occurred by giving an overdose of penthobarbital (24 mg/kg) followed by exsanguination. At slaughter, kidney, and diaphragmatical muscles and muscles at the injection place were sampled and tested the same day in the antigen ciELISA. The kidneys were tested simultaneously in the New Belgian Kidney Test (NBKT). In addition, all samples were stored at -20°C until analysis in a variant of the European Four-Plate Test (muscles and diaphragmatical muscles) and in the HPLC-UV (all tissues). Porcine kidney and muscle tissues free of any antimicrobial substance (tested compliant in the Belgian residue control program) were used as reference control samples. The HPLC-UV analysis and the microbiological assays were performed at the Department of Veterinary Public Health and Food Safety, Ghent University.

The samples (15 porcine or bovine kidneys) analysed in the antibody ciELISA, the Parallux™ assay and the NBKT were collected at slaughterhouses by the Belgian Institute for Veterinary Inspection and were stored at –20°C until analysed. The porcine kidneys used as

reference control samples in the antibody ciELISA were obtained at slaughter from animals reared on an experimental unit, without penicillin treatment.

Sample analysis in the antigen ciELISA

Sample preparation. The meat samples were cut into small pieces (0.5 cm^2) whereafter 4 g of sample was added to 18 ml extraction solution (Sodium phosphate buffer 0.1 M pH 5). After shaking briefly, 18 ml diluent (PBS + 6 % BSA + 0.1 % Tween 20®) was added. Subsequently, the samples were shaken and centrifuged (10 min, 12000 rpm or 17210 g). The supernatant was collected, filtered over Whatmanpaper no. 2 whereafter the pH was adjusted to pH 6.5-7 with 1N NaOH or HCl.

Sample analysis. The extracts were tested in the antigen ciELISA with pAb K2, as described in Chapter 5. A serial dilution of ampicillin in the extraction fluid of the corresponding control tissue was used as standard. The competition in the ELISA between free ampicillin in the sample and the coated ampicillin (ampMBSova) was calculated with the formula: competition (%) = $(1-(B/B_o))*100$ with B = absorbance of a tested sample solution and B_o the absorbance of a tested similar solution without a penicillin. The calibration curve was established by plotting the concentration of a standard dilution of ampicillin against the competition obtained for the ampicillin standard dilutions. The concentration of ampicillin in the samples could then be determined using the calibration curve.

Sample analysis in the antibody ciELISA

Sample preparation: tissue fluids were collected after centrifugation of 10 gram of minced tissue (10 min, 2000g). Kaolin (800 µl of 25% kaolin in PBS) was added to 100 µl tissue fluid. The mixtures were shaken thoroughly and incubated for 30 min at room temperature. After centrifugation (12000 g, 10 min), 650 µl of the supernatant was added to 350 µl PBS supplemented with bovine serum albumin (BSA, 6%) and Tween®20 (0.1%).

Sample analysis: the ELISA was performed as described (Cliquet et al., submitted). Test penicillins were hydrolyzed with Penicillinase I. Fluids from negative tissues and fluids from negative tissues spiked with amoxicillin at the MRL-value (50 ppb) were analysed as negative and positive control, respectively. A serial dilution of amoxicillin in the extraction fluid of the corresponding control tissue was used to construct a calibration curve (concentration vs competition). The competition in the ELISA between a free penicillin in the sample and the biotinylated ampicillin) was calculated with the formula: competition (%) =

$(1-(B/B_o))*100$ with B = absorbance of a tested sample solution and B_o the absorbance of a tested similar solution without a penicillin. The sample analyses were considered as valid, if the competition value for the positive control sample was in the range of the expected value (67%±4%). A sample was considered non-compliant when the concentration was higher than the detection capability (CCβ), compliant when lower than the decision limit (CCα) and suspected when between CCα and CCβ.

Repeatability of the ELISA

The repeatability of the antigen ciELISA (within-laboratory) was determined by repeating the analysis of ampicillin in buffer solution (8 different concentrations) six times on the same day and in the same assay (intra-assay variation), but also in six different assays (inter-assay variation) and by repeating the analysis on six different days (inter-day variation). The repeatability of the antibody ciELISA was determined similarly, but using hydrolysed amoxicillin.

Decision limit and detection capability of the ELISA

Control porcine tissue samples spiked with ampicillin (5 kidneys and 5 muscle tissues, antigen ciELISA) or amoxicillin (14 kidneys, antibody ciELISA) at the MRL (50 ppb) were analysed on different days. The average B/B_o for CCα and CCβ was calculated according to the Commission Decision 2002/657/EC. The corresponding concentrations were obtained by plotting the competition values (= $(1-B/B_o)*100$) in a calibration curve of ampicillin or amoxicillin in tissue extract (antigen ciELISA) or buffer solution (antibody ciELISA).

High Performance Liquid Chromatography (HPLC-UV)

The analyses were performed following the sample preparation procedure and HPLC method previously described (Boison et al., 1998). Briefly, the sample preparation occurred in three steps:

1) extraction of the penicillins with a phosphate buffer 0.1 M pH 9.2
2) clarification and concentration of the extract on a C18 column, eluted with acetonitril. After the acetonitril in the eluate was evaporated to dryness, the residue was dissolved in 200 µl acetonitril-phosphate buffer pH 6.5 (40:60, v/v).

3) precolumn derivatization of the penicillins in the extract by acetylation of the side chain amino group of ampicillin using benzoic anhydride followed by derivatization using mercuric chloride in presence of 1,2,4 triazole at 65°C for 30 min.

Nafcillin was included as internal standard. The extracts were analysed using HPLC with UV-photometrical detection (325 nm). A serial dilution of ampicillin in the extraction buffer was also analysed to determine a standard curve. Furthermore, the corresponding tissue control sample was spiked with ampicillin at the MRL-value (50 ppb) and was also analysed. The concentration of ampicillin in that spiked sample was then calculated using the standard curve and the recovery was determined. This factor and the standard curve are used to determine the concentration of ampicillin in the samples. A sample was considered non-compliant when the concentration of ampicillin was higher than 50 ppb.

The New Belgian Kidney Test (NBKT)

Paper disks (12.7 mm diameter) impregnated with kidney pelvis fluid and a piece of kidney cortex (a few mm) were incubated on an agar layer (pH 7.2) previously seeded with a susceptible bacterial strain (*Bacillus subtilis*). The growth inhibition zone was measured in millimeters. The samples were analysed twice. Samples producing an inhibition zone larger than 20 mm for the paper disk (zone inclusive the diameter of the disk) and larger than 2 mm around the cortex were considered as positive for the presence of antimicrobial substances.

Variant of the European Four-Plate test

Four agar plates were used: plate one was seeded with *Bacillus subtilis,* plate two with *Micrococcus luteus,* plate three with *E. coli* and plate four with *Bacillus cereus*. Penicillins can be detected on plate one and two. Plate three, seeded with *E. coli,* is more sensitive for the detection of quinolones and some cephalosporines. The fourth plate, seeded with *Bacillus cereus* is used for the detection of tetracyclines. Frozen pieces (disks, 8 mm diameter, 2 mm thick, approximately 100 µg) of muscles, liver and diaphragmatical muscle tissues were placed on the plates. The inhibition zone around the pieces was measured in millimeters. Samples producing an inhibition zone larger than 2 mm were considered as positive for the presence of antimicrobial substances. Inhibition zones larger than 12 mm correspond to standard buffer solution containing 3 ng ampicillin on the *B. subtilis* plate, and 0.8 ng ampicillin on the *M. luteus* plate. The presence of penicillins was confirmed by using

Penicillinase I (penase test). Hereto, the positive samples were retested on plate one and two, with and without paper disks impregnated with penicillinase, deposited at a distance of 2 mm from the samples. A penase test was recorded as positive when the inhibition zone was interrupted around the penase disk.

The Parallux™ assay (IDEXX Laboratories)

Assay design: the method is a competitive fluorescent immunoassays, simultaneously carried out in 4 capillary tubes on a cartridge. For the detection of β-lactams, ampicillin, amoxicillin and benzylpenicillin, and in a lesser extent also cloxacillin and dicloxacillin, are detected in the first capillary, cephapirin in the second, ceftiofur in the third and finally, cloxacillin and dicloxacillin in the last tube. The fluorescent signals are measured and reported by the processor of the Parallux™ device within 5 minutes.

Sample preparation: Five grams of partially thawed tissue are diluted with 10 ml of phosphate buffer pH 7.6, and mixed using a stomacher. Two ml of the dilution are centrifuged at 20000 g for 5 min. The supernatants are ready for analysis in the Parallux™ device.

Sample analysis: the extract (100 µL) is added to the reagent tray containing the fluorescent-labelled antibodies. Sample and reagents are automatically mixed and transferred to the cartridge capillary tubes coated with antigen. Penicillin in the sample will bind to the labelled antibodies. The unbound antibodies will bind to the immobilized antigen. After a short incubation, the sample penicillin–antibody complexes are washed away and the fluorescent signal of the antibodies bound on the coated antigen is measured. A test sample was considered non-compliant if the signal obtained for the kidney sample was higher than 1.50 (Okerman et al., 2003).

Results and discussion

Specificity and limit of detection (LOD) of the ELISAs

The polyclonal antibodies pAb K2 are highly specific for penicillins (Cliquet et al., submitted). No cross-reactions were observed for cephadrin, cephalexin, cefazolin, sulfanilamide or chloramphenicol analysed in the antibody ciELISA (without hydrolysis). Low cross-reactivities were only noticed for cefaclor when analysed at high concentration (concentration cefaclor for which the binding of the antibodies is inhibited for 50% (IC50) = 5000 ppb) compared to the penicillins. The cross-reactivities within the group of penicillins

and the detection limit for the different penicillins in buffer solutions in the antigen ciELISA as well as in the antibody ciELISA using pAb K2 are described in Chapter 5 (Cliquet et al., submitted). In the antigen ciELISA, only ampicillin is detected below the MRL (LOD = 6 ppb). With the antibody ciELISA, it is possible to detect the tested hydrolysed penicillins at concentrations below the MRL. The ELISA is most sensitive for ampicillin (LOD < 0.1 ppb), followed by amoxicillin (LOD < 1 ppb), benzylpenicillin (LOD = 1 ppb) and oxacillin (LOD = 0.6 ppb). Although in a lesser extent, cloxacillin and dicloxacillin are also detected below the MRL (LOD = 7 and 30 ppb, respectively).

During the analysis of samples, a reference penicillin is also analysed. Decision of compliance for samples should be made in regard to that reference penicillin. The antigen ciELISA is only suitable for the detection of ampicillin below the MRL and therefore ampicillin is also used as reference. With the antibody ciELISA, all tested penicillins can be detected below the MRL. Ampicillin is not a suitable reference in this case because the detection is at least ten times more sensitive as compared to the other penicillins. Therefore amoxicillin was chosen as reference penicillin. Consequently, the validation characteristics were determined for the reference penicillin.

Repeatability of the ELISA

The highest within-laboratory variation observed was seen in the same assay and was 15% for the antibody ciELISA (Table 7.1) and 14 % for the antigen ciELISA (Table 7.2). For immunoassays, the coefficient of variation should not exceed 15 % (Crabbe, 2002). The inter-laboratory variation was not assessed.

Table 7.1: Repeatability (Coefficient of variation, %) of the antibody ciELISA with pAb K2.

	Coefficient of variation (%) for amoxicillin (ppb)					
	40	20	10	5	2.5	1.25
Intra-assay	5-11	5-12	1-13	5-15	6-12	6-14
Inter-assay	0	1	1	2	4	7
Inter day	1	1	2	4	3	4

Table 7.2: Repeatability (Coefficient of variation, %) of the antigen ciELISA with pAb K2.

	Coefficient of variation (%) for ampicilline (ppb)							
	5000	1000	500	100	50	10	5	2.5
Intra-assay	1-2	2-3	1-14	2-9	2-7	1-8	2-7	1-4
Inter-assay	5	7	6	6	13	5	5	4
Inter day	14	9	8	7	7	6	8	5

Sample preparation

Two different sample preparations were applied for the analysis in the antigen ciELISA and in the antibody ciELISA. The antigen ciELISA and corresponding extraction procedure using meat tissue were developed before the antibody ciELISA. The latter one was more sensitive for the detection of penicillins and therefore it was decided to look for a simple sample preparation using meat fluids only for the antibody ciELISA. The use of meat fluids was thus not investigated for analysis in the antigen ciELISA. Because this sample preparation was very satisfying for the antibody ciELISA, the previously developed extraction procedure using meat tissue was not applied for analysis in the antibody ciELISA.

Usually, tissue samples are minced with a conventional kitchen mixer before adding extraction buffer (Mc Cracken et al., 2000). This procedure however, led to high background signals in the antigen ciELISA. Therefore, the samples where cut into small pieces and subsequently mixed with extraction buffer. Interestingly is the matrix effect of porcine kidney and muscle tissues on the detection of ampicillin in the antigen ciELISA. The LOD for ampicillin in porcine kidney or muscle extracts is lower (1 ppb and <1 ppb, resp.; Table 7.3) than the LOD for buffer solution (6 ppb, Cliquet et al., submitted). Mostly, higher LODs are reported for tissue samples as compared to buffer solutions (Okerman et al., 1998a). Apparently, the kidney and muscle matrix favourably influenced the detection of ampicillin in the antigen ciELISA. Moreover, a calibration curve built up by spiked muscle or kidney samples is identically to a calibration curve built up by spiked extraction fluids of the respectively tissues. This means that ampicillin is extracted from spiked samples with 100 % recovery. Therefore, when analyzing incurred samples in the antigen ciELISA, ampicillin dilutions in extracts are used as reference.

Penicillins are present in the intercellular fluid of tissues (Divers, 1996). Therefore, tissue fluids were used for analyzing samples in the antibody ciELISA. The use of tissue fluid simplifies the sample preparation and analysis. Before analysis in ELISA, the fluids were treated with kaolin. Kaolin treatment reduces the background signals in ELISA by removing

disturbing fat or other matrix components (Van den Broeck et al, 1999). Contrary to the extraction procedure applied for the analysis in the antigen ciELISA, a calibration curve built up by spiked kidney tissues is identically to a calibration curve built up by buffer solutions (Figure 7.2). Therefore, when analyzing incurred samples in the antibody ciELISA, buffer solutions of amoxicillin are used as reference. The LOD for amoxicillin in buffer solutions or kidney tissues is lower than 1 ppb (Table 7.3).

During both extraction procedures, samples are diluted ten times. As a result, the ELISA must be at least ten times more sensitive than the MRL. This requirement is thus fulfilled for both ELISAs.

Decision limit and detection capability

The decision limit (CCα) in the case of substances with a permitted limit (MRL) is the limit at and above which can be concluded with an error probability of 1-α that a sample is non-compliant. The α-error is the probability to obtain a non-compliant result for a sample that is truly compliant (false non-compliant). The detection capability (CCβ) is the concentration at which a method is able to detect permitted limit levels with a statistical certainty of 1-β. The β–error is the probability that a sample is considered compliant while being truly non-compliant (false-compliant result) (Anonymous, 2002). According to the Commission Decision 2002/657/EC, at least 20 samples should be analysed to determine CCα and CCβ. The more samples are analysed, the more precise will be the estimation of CCα and CCβ. In this study, the sample analysis for the antigen ciELISA and the antibody ciELISA were performed in 1999 and 2001, respectively, and thus before publication of the Commission Decision. Consequently, CCα and CCβ were calculated using the available data (5 kidneys and 5 livers for the antigen ciELISA, 14 kidneys for the antibody ciELISA). The resulting values are therefore an estimation of CCα and CCβ.

A sample is considered non-compliant when the concentration is higher than CCβ, compliant when lower than CCα and suspected when between CCα and CCβ. The range between CCα and CCβ is larger for kidneys (21-69 ppb) as for muscle samples (8 – 21 ppb) in the antigen ciELISA (Table 7.3). This means that analysis of muscle samples is more precise than the analysis of kidney samples in the antigen ciELISA. The precision of the kidney analysis in the antibody ciELISA is comparable to the muscle analysis in the antigen ciELISA (Table 7.3).

Table 7.3: Decision limit (CCα), detection capability (CCβ) and limit of detection (LOD) for ampicillin in muscle and kidney samples analysed in the antigen ciELISA, and for amoxicillin in kidney samples analysed in the antibody ciELISA.

		Antigen ciELISA		Antibody ciELISA
Penicillin		Ampicillin		Amoxicillin
Matrix		Muscle	Kidney	Kidney
X_{MRL}	Number of samples	5	5	14
	B/B_o[1] (average ± SD)	0.8780 ± 0.0327	0.8392 ± 0.0459	0.3300 ± 0.0380
	Competition (average ± SD)	12 % ± 3 %	16 % ± 5 %	67 % ± 4 %
	Concentration[4]	5 ppb	5 ppb	5 ppb
CCα	B/B_o[2]	0.8243	0.7639	0.2676
	Competition	18 %	23 %	73 %
	Concentration[4]	8 ppb	21 ppb	9 ppb
CCβ	B/B_o[3]	0.7707	0.6886	0.2052
	Competition	23 %	31 %	79 %
	Concentration[4]	21 ppb	69 ppb	19 ppb
LOD	Concentration	< 1 ppb	1 ppb	< 1 ppb (< 56%)

[1] B/B_o (X_{MRL})= average absorbance of the sample / average absorbance of the control tissue samples
[2] B/B_o (CCα) = $B/B_o(X_{MRL})$ – 1.64* SD, (Anonymous, 2002)
[3] B/B_o (CCβ) = B/B_o(CCα) – 1.64* SD, (Anonymous, 2002)
[4] Concentration calculated using the corresponding calibration curve (competition *versus* concentration)

The lower precision of the kidney analysis in the antigen ciELISA can be ascribed to two factors. Firstly, the variation (standard deviation, SD) on the average X_{MRL} is slightly larger for kidney as compared to muscle tissues. Consequently, larger differences between the calculated CCα and CCβ are noticed. Secondly, the flatness of the calibration curve for muscle and kidney samples in the antigen ciELISA has as consequence that little variations in the y-value (signal or competition, Figure 7.1) cause large differences in the extrapolated x-value (concentrations) (Tijssen, 1985). The calibration curve of the antibody ciELISA is steeper, especially in the region around the MRL (Figure 7.2). Consequently, small differences in signal correspond to small differences in concentrations, and large differences in signal correspond to large differences in concentrations. Such a calibration curve combined with a high repeatability and thus small variation, provide analyses of samples with high precision.

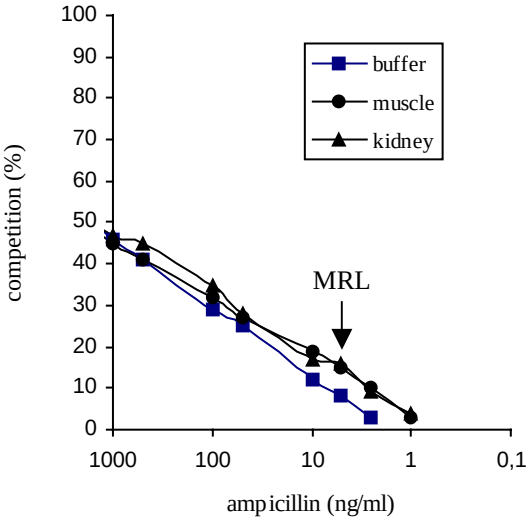


Figure 7.1: Calibration curve of ampicillin in buffer solution, porcine muscle and kidney extract, analysed in the antigen ciELISA with pAb K2

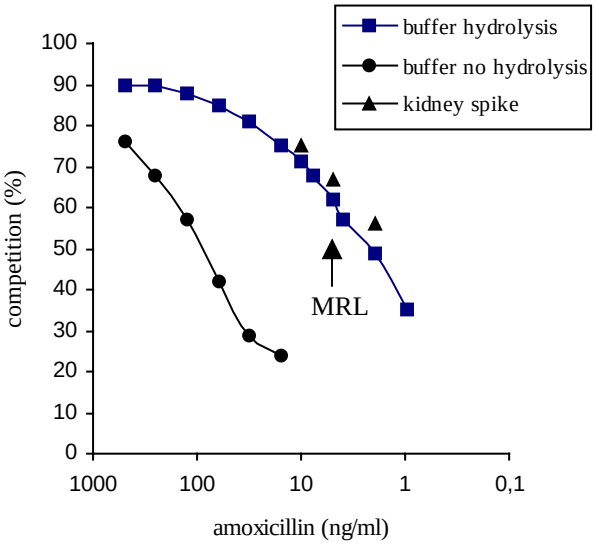


Figure 7.2: Calibration curve of amoxicillin in buffer solution (hydrolysed or not) and spiked porcine kidney samples (hydrolysed), analysed in the antibody ciELISA with pAb K2

Analysis of incurred samples in the antigen and antibody ciELISA

To determine the detection capability of the ELISA in accordance to the Commission Decision 2002/657/EC, spiked samples containing penicillin at the MRL should be used. Spiked samples are more appropriate for that purpose than samples obtained from *in vivo* experiments because it is difficult to predict the concentration that will be obtained in the tissues after administration of the drug to animals. On the other hand, care must be taken when validating a method using spiked samples. An extraction protocol optimized using spiked samples will not necessarily be efficient for incurred samples. The efficiency of the extraction procedure to extract analytes from the tissue matrix cannot be determined using spiked samples because the interaction between analyte and the sample matrix is different for incurred samples (Mc Cracken et al., 2000). Therefore, the analysis of incurred samples in the antigen and in the antibody ciELISA was compared to the analysis of the same samples using other qualitative or quantitative methods.

The antigen ciELISA versus HPLC-UV and microbiological assays

Twelve pigs were treated with ampicillin and slaughtered at different time points during the withdrawal period. The kidney and muscle tissues (at the injection place and diaphragmatical muscle tissues) were analysed in the antigen ciELISA for the presence of ampicillin. The results were compared following the analysis of the same samples using a

physicochemical method (HPLC-UV) and two microbiological assays (variant of the Four-Plate test and the NBKT). The results of the analysis of muscles (injection side), kidneys, and diaphragmatical muscles using the antigen ciELISA and using the three other methods are shown in Table 7.4, 7.5 and 7.6, respectively.

Table 7.4: Comparison of the analysis of the muscles (injection side) with the 4 methods.

Pig no.	Dose	Euthanasia[1]	Muscle				
	(mg/kg)	(hours)	ELISA (µg/kg)	Micro Bs[2] (mm)	Micro Ml (mm)		HPLC-UV (µg/kg)
					no penase[3]	penase[4]	
7	3.75	48	0	<2/<2	10.0/6.3	3.8/3.2	33 ± 6
10	3.75	48	84.8	<2/<2	4.7/3.0	<2/<2	30 ± 7
1	7.5	48	62.2	5.3/7.0	>12/>12	<2/<2	483 ± 155
8	7.5	48	450	3.7/<2	>12/>12	<2/<2	119 ± 23
2	15	48	35.3	<2/<2	>12/>12	<2/<2	61 ± 11
4	15	48	1.1	<2/<2	4.2/4.5	<2/<2	46 ± 4
13	15	24	190	>12/>12	>12/>12	<2/<2	234 ± 64
14	15	24	2723	7.1/7.2	>12/>12	8.5/<2	118 ± 25
20	15	4	>10000	>12/>12	>12/>12	ND[5]	>1000
22	15	4	>10000	>12/>12	>12/>12	ND	>1000
16	15	2	>10000	>12/>12	>12/>12	ND	>1000
19	15	2	>10000	>12/>12	>12/>12	<2/<2	>1000

[1]time after 2de injection
[2]Micro Bs = microbiological assay using *Bacillus subtilis*
[3]Micro Ml no penase= microbiological assay using *Micrococcus luteus*
[4]Micro Ml penase= microbiological assay using *Micrococcus luteus* and Penicillinase I
[5]ND = no data

Table 7.5: Comparison of the analysis of the kidneys with the ELISA, the NBKT and the HPLC-UV.

Pig no.	Dose	Euthanasia[1]	Kidney			
	(mg/kg)	(hours)	ELISA (µg/kg)	NBKT Paper disk (mm)	NBKT Cortex (mm)	HPLC-UV (µg/kg)
7	3.75	48	2.6	14.75	<2	0
10	3.75	48	13.6	20.4	<2	0
1	7.5	48	12.3	<12.7	<2	0
8	7.5	48	40.5	12.7	<2	0
2	15	48	10.3	<12.7	<2	0
4	15	48	10.2	<12.7	<2	0
13	15	24	1100.0	19.35	<2	0
14	15	24	18.1	18.15	<2	0
20	15	4	>10000	42.15	8.7	>1000
22	15	4	>10000	40.35	8.9	>1000
16	15	2	>10000	48.1	12.1	>1000
19	15	2	>10000	48	12	>1000

[1]time after 2de injection

Table 7.6: Analysis of the diaphragmatical muscle tissues with the ELISA, the microbiological assay using *B. subtilis* and the HPLC-UV.

Pig no.	Dose	Euthanasia[1]	Diaphragmatical muscle			
	(mg/kg)	(hours)	ELISA (µg/kg)	Micro Bs (mm)		HPLC-UV (µg/kg)
				No penase[2]	Penase[3]	
7	3.75	48	0,0	<2/<2		0
10	3.75	48	0.0	<2/<2		0
1	7.5	48	19.0	<2/<2		0
8	7.5	48	4.2	<2/<2		0
2	15	48	12.8	<2/<2		0
4	15	48	0.0	<2/<2		0
13	15	24	1480.0	8/>12	<2/<2	0
14	15	24	509.0	<2/<2		0
20	15	4	907.0	8.9/9.6	<2/<2	>1000
22	15	4	46.0	6.5/9	<2/<2	>1000
16	15	2	459.0	10/10.4	<2/<2	>1000
19	15	2	347.0	11.3/10.6	<2/<2	>1000

[1]time after 2de injection
[2]Micro Bs no penase= microbiological assay using *Bacillus subtilis*
[3]Micro Bs penase= microbiological assay using *Bacillus subtilis* and Penicillinase I

A comparison was made between the ELISA and HPLC-UV for the quantitative analysis of the samples. HPLC-UV is a chemical analytical method combining liquid chromatography with UV-detection and was used for the detection of penicillins before the introduction of the LC-MS/MS method (Boison, 1995; Blanchflower et al., 1994). The disadvantage of the HPLC-UV method is the need of a derivatization of the penicillins using toxic reagents to allow detection because penicillins do not have any specific strong ultraviolet (UV) absorption (Ito et al., 1999). The concentrations of penicillins determined by HPLC-UV did not match with the concentrations determined with the ELISA (Table 7.4, 7.5, 7.6). When comparing two methods, care must be taken that the same metabolite of the analyte is detected. The ELISA does not discriminate between native and hydrolysed ampicillin (Cliquet et al., submitted), whereas only the native molecule is detected in HPLC-UV (Boison, 1995; Blanchflower et al., 1994). Moreover, the ratio hydrolysed/native penicillins in a sample is not equal for all samples and thus no correlation using a conversion factor can be made between both methods. Another difference between both methods is the extraction procedure. A phosphate buffer with pH 5 is used for the analysis in ELISA as compared to a phosphate buffer with pH 9 for HPLC-UV. The lower the pH of an extraction buffer, the more water is extracted from the tissue matrix. Penicillins are present in the intercellular fluid of tissues (Divers, 1996). Thus, it is possible that more penicillins are

extracted using the ELISA extraction procedure as compared to the one used for HPLC-UV analysis. All these factors could explain the different concentrations and made that the precision and efficiency of the ELISA could not be based on a quantitative comparison of both methods.

For the qualitative analysis of the samples, a comparison was made between the ELISA, the HPLC-UV and microbiological assays. The NBKT and the variant of the European Four-Plate test are bacterial inhibition assays and are qualitative tests (Okerman, 1995; Okerman et al., 1998b; Okerman et al., 1999). When a sample is found positive, it only indicates the presence of an antimicrobial agent. However, in the present study pigs were only treated with ampicillin. Therefore a positive result indicates the presence of ampicillin in the sample. When the same sample was found negative in the HPLC-UV analysis, this proves that the HPLC-UV produced false compliant results. Therefore, a sample found positive in the microbiological assay but negative in HPLC-UV should be considered non-compliant. On the other hand, HPLC-UV analysis could only detect ampicillin. Therefore, a sample found positive in HPLC-UV but negative in the microbiological assay was also considered non-compliant. Taking this into account, both assays together had to be used as reference method. The sensitivity and specificity of the ELISA in regard to the other methods were determined to obtain information on the correlation between ELISA and these methods. For the ELISA, samples were considered positive (non-compliant) if the concentration was higher than CCβ. Doing this, two of the 36 samples were positive in ELISA but negative (compliant) in the reference method, and three samples were negative in ELISA but positive (non-compliant) in the reference method (Table 7.7). The sensitivity of the ELISA was 0.82 and the specificity was 0.89.

Table 7.7: Determination of the sensitivity and specificity of the antigen ciELISA (in regard to CCβ).

		Reference method: HPLC-UV and microbiological assay	
		Non-compliant	Compliant
Method to validate: ELISA	Positive	a = 14	b = 2
	Negative	c = 3	d = 17

N = total amount of samples analysed = a+b+c+d
a+c = total amount of non-compliant (really positive) samples
b+d = total amount of compliant (really negative) samples
Sensitivity = a / (a + c) = 0.82
Specificity = d / (b + d) = 0.89

However, since CCβ was not determined for the HPLC-UV and the microbiological assays, the positive/negative estimations for these methods were done in regard to a positive control sample spiked at the MRL (HPLC) or in regard to a given inhibition zone (20 mm for the NBKT, 2 mm for the four-plate assay). If the ELISA results are estimated in the same manner (positive when concentration higher than 50 ppb instead of CCβ), a sensitivity of 0.95 and specificity of 0.94 is obtained (Table 7.8).

Table 7.8: Determination of the sensitivity and specificity of the antigen ciELISA (in regard to the MRL).

		Reference method: HPLC-UV and microbiological assay	
		Non-compliant	Compliant
Method to validate: ELISA	Positive	a = 18	b = 1
	Negative	c = 1	d = 16

N = total amount of samples analysed = a+b+c+d
a+c = total amount of non-compliant (really positive) samples
b+d = total amount of compliant (really negative) samples
Sensitivity = a / (a + c) = 0.95
Specificity = d / (b + d) = 0.94

According to the Commission Decision 2002/657/EC, the sensitivity or the capability of a screening assay to detect non-compliant samples as really non-compliant, should be at least 0.95 (β–error = 5%). So, based on the first calculation (sensitivity = 0.82), the ELISA procedure should be rejected or further optimized. However, based on the second calculation (sensitivity = 0.95), the ELISA meets the requirement of the Commission Decision 2002/657/EC and could thus be applied for screening purposes of ampicillin in porcine kidney and muscle tissues. However, none of both calculations are correct because the methods are not compared using the same parameters. As for the comparison of the quantitative analysis, these results demonstrate that it is difficult to compare results obtained with different methods validated using different parameters. It also demonstrates that comparison should be made in regard to a validated physico-chemical reference method or a similar, but also validated immunochemical method.

The antibody ciELISA versus microbiological assay and Parallux™ assay

As for the antigen ciELISA, the efficiency of the extraction procedure and analysis of samples in the antibody ciELISA was assessed with incurred samples. Porcine and bovine

kidney samples were obtained from the Belgian monitoring program and analysed in the antibody ciELISA, the NBKT and the Parallux™ assay.

The Parallux™ is a commercial screening assay for the detection of β-lactam antibiotics in milk (Huth et al., 2002). Although developed for the detection of antibiotics in milk, the Parallux™ assay was found suitable for the qualitative screening of different antibiotics (tetracyclins, β-lactams and cephalosporins) in porcine and bovine kidneys (Okerman et al., 2003). The Parallux™ and ELISA detect ampicillin, amoxicillin, benzylpenicillin, cloxacillin and dicloxacillin. The ELISA also recognizes oxacillin. Amoxicillin was chosen as standard penicillin in the ELISA. The detection capability and decision limit were established for that standard penicillin. However, the different penicillins are detected with different sensitivities (different cross-reactivities) in the ELISA. Consequently, CCβ will be different for each penicillin. If a sample is positive during screening, the identity of the penicillin in the sample is not known. Therefore, for multi-analyte methods, decision based on CCβ will not necessarily give certainty about the false compliant rate of the ELISA.

The results of the analysis of incurred samples in the NBKT, the Parallux™ assay and the ELISA are given in Table 7.9. Not only penicillins, but also other substances with antimicrobial activity are detected in the NBKT. Because the incurred samples came from animals with unknown treatment, a positive result in this test indicates the presence of one or more antimicrobial substances and thus not only penicillins. This is the reason why some samples (sample n° 407, 454) are tested positive in the NBKT, but negative in the Parallux™ and the ELISA. High correlation (r^2 = 0.94) was found between the Parallux™ and the ELISA. Only sample n°1199 was tested negative for the presence of penicillins in the Parallux™ while positive in the NBKT and slightly positive in the ELISA (although far below CCα, 73%). However, it was found positive for ceftiofur in the Parallux™. It is possible that the ELISA also detected ceftiofur instead of penicillins. The ELISA shows some cross-reactivity toward the structural related cefaclor (Cliquet et al., submitted). However, cross-reactivity for ceftiofur was not tested.

Table 7.9: Results of the analysis of incurred kidney samples in the NBKT, the Parallux™ and the antibody ciELISA

Sample N°	NBKT Cortex (mm)	Parallux™ Signal	ELISA Competition (%)
1508	NT[1]	2	74
1770	>2/>2	2	72
1703	2/2	0	0
1390	>2/<2	2.03	62
1109	>2/>2	2.48	76
407	>2/>2	0	0
1199	4.5/7.6	ceft[2]	12
1388	>2/>2	2.3	73
1393	<2/<2	2.07	55
1511	>2/>2	2.63	66
1408	<2/<2	0	0
1332	<2/<2	2.57	66
1679	> 2/ >2	2.5	74
1329	<2/<2	0.77	30
454	>2/>2	0	0

[1]NT = not tested
[2]ceft: The suspected analyte was identified as ceftiofur with LC-MS/MS

Conclusion

The antibody ciELISA using pAb K2 is preferred above the antigen ciELISA with pAb K2 because the detection limit of the antibody ciELISA for penicillins is far below the MRL. Furthermore, the precision of the antibody ciELISA is higher than the antigen ciELISA: the range between CCα and CCβ is smaller for the analysis of kidney samples in the antibody ciELISA as compared to the antigen ciELISA. Moreover, the suitability of the ELISA and extraction procedure for screening purposes was demonstrated by comparing the analysis of incurred samples. Almost 100 % correlation was found between the ELISA and a commercial immunochemical method, the Parallux™ assay.

Acknowledgements: This study is supported by the Ministry of Agriculture, Brussels, Belgium and by the Institute of Veterinary Inspection (IVK). The technical assistance of Els De Vogelaere was greatly appreciated.

Part IV

Immunochemical detection of sulfonamides

Chapter 8

Generation of group-specific antibodies against sulfonamides

Based on: P. Cliquet, E. Cox, W. Haasnoot, E. Schacht and B. M. Goddeeris. Generation of group-specific antibodies against sulfonamides. J. Agric. Food Chem., 2003,51, 5835-5842

Abstract

To develop a sulfonamide specific ELISA, different attempts were made to obtain monoclonal antibodies specific for the common structure of sulfonamides. In a first approach, sulfanilamide was linked to albumins using glutaraldehyde or a succinimide ester as cross-linker. No or a weak immune response was induced after immunization of mice with these conjugates. High antibody titers were obtained with conjugates where sulfanilamide was linked to albumins or casein (azocasein) with a diazotation reaction. However, the antibodies were only highly specific for the bound sulfanilamide molecule. In a second approach, sulfonamide-protein conjugates were used in which the sulfonamide molecule is linked at its side chain, leaving the common structure of sulfonamides unchanged. Three sulfonamide derivatives (S, TS and PS; previously described in literature) containing a carboxyl group in their side chain were linked to proteins using a carbodiimide mediated reaction. Immunization with the S-conjugates led to high antibody titers, but the antibodies were only highly specific for the bound S-molecule. Group-specific antibodies were obtained after immunization with the PS- and TS-conjugates. It was described that immunization with PS-conjugates lead to the recognition of other sulfonamides (sulfamethazine, -merazine, -diazine and –dimethoxine) that are not well recognized by antibodies induced after immunization with TS-conjugates. Therefore, we tried to guide the immune response in the direction of recognition of the common structure of sulfonamides by immunizing the animals alternately with PS- and TS-conjugates. The polyclonal antibodies of the mice had indeed a broader specificity, but the specificity of the monoclonals obtained after fusion experiments was not influenced. Immunization with TS-conjugates seemed sufficient to obtain sulfonamide-specific monoclonal antibodies. With the best monoclonal (mAb 3B5B10E3) two competitive inhibition (ci) ELISAs were developed: one coated with antigen and the other coated with the monoclonal antibody. Sulfadiazine, –dimethoxine, -thiazole, -pyridine and -methoxazole were detected in both ELISAs at their MRL-value (100 ppb) in buffer solution. Sulfadiazine, sulfathiazole and sulfamethoxazole could even be detected at 10 ppb.

Keywords: Sulfonamide – monoclonal antibody - ELISA

Introduction

Sulfonamides are chemotherapeutical reagents widely used in human as well as in veterinary medicine for the treatment of bacterial infections. They are also used as growth promoting feed additives (Long et al., 1990). As a consequence, sulfonamides have appeared in food products from animal origin (Franco et al., 1990). To protect consumers from risks related to drug residues, maximum residue limits (MRL) are determined by law. In Europe, Canada and the United States, the MRL for total sulfonamides in edible tissues and milk is 100 ppb (Anonymous, 1999; Anonymous, 1991), whereas it is 20 ppb in Japan. In Europe, at least nine sulfonamides are allowed for use in veterinary medicine (sulfamethazine, sulfadiazine, sulfadimethoxine, sulfanilamide, sulfathiazole, sulfadoxine, sulfamethoxazole, sulfatroxazole, sulfachloropyridazine…). Screening methods for sulfonamides include bioassays, immunoassays, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC, LC-MS/MS). Liquid chromatography is a sensitive and specific assay but is also very laborious and expensive. The method is more suitable for confirmation than for screening of large amounts of samples. A rapid, sensitive and specific assay is needed to pick up positive samples in routine analyses, which then can be confirmed for the presence of sulfonamides by liquid chromatography. Therefore, during the past ten years, a variety of immunoassays were developed, each highly specific for an individual sulfonamide (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Sheth and Sporns, 1990; Garden and Sporns, 1994; Muldoon et al., 2000; Lee et al., 2001; Spinks et al., 2001). However, it would be more efficient to have one immunoassay able to detect all sulfonamides instead of several immunoassays, each specific for an individual sulfonamide.

The sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the *N*4-position and differ in the substitution at the *N*1-position (Figure 3.1, chapter 3). For the group-specific detection of sulfonamides, antibodies against the aromatic amino group are needed. Sheth and Sporns (1991) were the first who reported the development of sulfonamide-specific antibodies. They immunized rabbits with a sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]-sulfonamide, TS) linked at its side chain to limulus polyphemus haemocyanin (TS-LPH). The polyclonals recognized nine sulfonamides showing 50 % inhibition at a concentration of less than 5 µg/mL. Assil et al. (1992) synthesized another sulfonamide derivative with a larger side chain (N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide, PS). The polyclonal serum

they obtained was purified by affinity chromatography and the purified fraction showed 50 % inhibition with seven sulfonamides at concentrations less than 10 µg/mL. The first published study about sulfonamide-specific monoclonal antibodies (mAb) was from Muldoon et al. (1999). After immunization with a N-sulfanilyl-4-aminobenzoic acid-protein conjugate, only one mAb was obtained that recognized eight sulfonamides at levels less than 10 µg/mL. Haasnoot et al. (2000a; 2000b) used the sulfonamide derivatives of Sheth and Sporns (1991) and Assil et al. (1992) to induce mAb. The best mAb showed 50 % inhibition with 18 tested sulfonamides at values less than 10 µg/mL, and with eight at a concentration of less than 0.1 µg/mL. Unfortunately, the relevant sulfonamides sulfamethazine, sulfatroxazole and sulfachloropyrazine were not detected at the MRL value (100 ppb). Spinks et al. (1999) carried out molecular modeling studies on the sulfonamide structure revealing that the molecule has a characteristic bend around the tetrahedral $–SO_2-$ grouping. Recognition of the common structure would be maximal in these drugs where the bend had the greatest angle. They deduced that cross-reactive antibodies could possibly be obtained using a sulfonamide as hapten with a more planar structure (sulfacetamide) or a greater bend (sulfachloropyridazine). Despite this interesting hypothesis, immunization with such conjugates did not lead to antiserum with a broad specificity for sulfonamides. Finally, Li et al. (2000) reported the detection of sulfonamides in swine meat by immunoaffinity chromatography using cross-reactive polyclonal antibodies (pAb) induced with three different sulfonamide haptens: N1-(4-carboxyphenyl)sulfanilamide (H1), N1-(4-carboxyphenyl)-N4-(4-aminobenzenesulfonyl)sulfanilamide (H2) and N1-(6-carboxyhexyl)sulfanilamide (H3). Sulfonamides were best recognized by the antibodies induced with H2-protein conjugates, slightly lesser with H1-protein and worst with H3-protein conjugates. None of the reported mAbs or pAbs (except these of Li et al. (2000), which cross-reactivity values were not mentioned) were able to detect all of the relevant sulfonamides (sulfamethazine, -doxine, -chloropyridazine, -quinoxaline, sulfatroxazole, …) at the MRL.

In this study, different strategies for the development of sulfonamide-specific mouse antibodies are compared. In a first approach, sulfanilamide was chosen for the construction of hapten-protein conjugates because this molecule is the common structure of the sulfonamides. Antibodies against sulfanilamide should therefore be group-specific. The conjugations were achieved using glutaraldehyde or succinimide ester as cross-linker, or using a diazotation reaction. In a second approach, sulfonamide-protein conjugates were used in which the sulfonamide molecule is linked at its side chain, leaving the common structure of

sulfonamides unchanged and thus free for the induction of group-specific antibodies. In previous studies it was demonstrated that the specificity of antibodies obtained after immunization with PS-protein conjugates was different from the specificity of antibodies induced with TS-protein conjugates (Haasnoot et al., 2000a; Haasnoot et al., 2000b). Therefore, mice were immunized alternately with PS- and TS-conjugates in order to induce antibodies with a broader specificity.

Material and methods

Reagents and chemicals

Sulfamethazine, sulfamerazine, sulfathiazole, sulfadiazine, sulfadimethoxine, sulfamethoxazole, sulfachloropyridazine, sulfapyridine, sulfisoxazole, sulfanilamide, para-aminobenzoic acid, bovine serum albumin (bsa), thyroglobulin, ovalbumin (ova), 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC), *s*-acetylmercaptosuccinic anhydride (SAMSA), 3-maleimidobenzoic-*N*-hydroxysuccinimide ester (MBS), Ellman's reagent (= 5,5'-dithio-bis(2-nitrobenzoic acid), citraconic anhydride, bicinchoninic acid (BCA), cupper(II)sulphate pentahydrate ($CuSO_4.5H_2O$), kaolin, OPI supplement media and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Bornem, Belgium). Furosemide, acetazolamide, hydrochlorothiazide, bumetanide, thiamphenicol, florphenicol and lidocaine were a kindly gift of the Department of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Ghent University. The TMB substrate solution was prepared by adding 3.3 mg TMB in 250 µL DMSO to 25 mL phosphate-citrate buffer (0.1M citric acid + 0.2M Na_2HPO_4; pH 4.3) containing 3.25 µL of a 30 % H_2O_2 solution. Biotin-LC-PEO-amine and keyhole lympet hemocyanin (klh) were purchased from Pierce, (Perbio, Erembodegem-Aalst, Belgium). Polyethylene glycol 1500 (PEG), ABTS (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) tablet® and ABTS buffer® were obtained from Roche Diagnostics (Brussels, Belgium). The ABTS substrate solution was prepared by dissolving 1 ABTS tablet® (5 mg) in 50 mL ABTS buffer®. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were provided by Difco Laboratories (Biotrading, Bierbeek, Belgium). Tween®20 (polyoxyethylene sorbitan monolaurate) and Tween®80 (polyoxyethylene sorbitan monooleate) were purchased from Merck-Belgolabo (Overijse, Belgium). Rabbit anti-mouse immunoglobulins (code n° Z0259) and rabbit anti-mouse immunoglobulins conjugated to peroxidase (code n°P0260) were obtained from DAKO

Diagnostica (Prosan, Ghent, Belgium). Dialysis tube VIKING (12 000 –14 000 MW cut off) was provided by ROTH (Fiers, Kuurne, Belgium). Dimethylformamide (DMF) was supplied by SERVA (Polylab, Antwerp, Belgium). Dimethylsulfoxide (DMSO) was from VWR (Leuven, Belgium), ELISA microtiter plates (maxisorp) from NUNC (Life technologies, Merelbeke, Belgium), tissue culture plates from Greiner (Wemmel, Belgium). Dulbecco modified Eagle's medium (DMEM), glutamine, gentamycin, sodium pyruvate, foetal calf serum (FCS) and hypoxanthine, aminopterin and thymidine supplement (HAT) were purchased from GibcoBRL (Life technologies, Merelbeke, Belgium). All other chemicals were of reagent grade or better. HAT-selection medium consisted of DMEM containing 20% FCS, 1% glutamine, 0.1% gentamycin, 1% sodium pyruvate, 1% OPI supplement media and 2% HAT.

Commercially available sulfonamide-protein conjugates

Azocaseïne was purchased from Sigma-Aldrich (Bornem, Belgium), sulfamethazine-bovine gamma globulin antigen conjugate (smt-bgg) was obtained from Chemicon International INC. (Biognost, Wevelgem, Belgium).

Synthesis of sulfanilamide-protein conjugates

Conjugation by diazotation: sulfanilamide was conjugated to bsa according to Fleeker and Lovett (1985). Briefly, sulfanilamide (34.5 mg) was diluted in 0.5 N H_2SO_4 by heating. After cooling, 1 mL $NaNO_2$ (19 mg/mL) was added over 3 min. The solution was stirred for another five minutes whereafter it was added over 10 min to a cooled bsa solution (100 mg in 4 mL sodium carbonate 1M pH 10) and subsequently incubated during 4 h at room temperature: sulfa-bsa.

Conjugation with glutaraldehyde according to Van Regenmortel et al. (1988). Briefly, 7.6 mg sulfanilamide was diluted in 1 mL PBS and added to 5 mL ova solution (1 mg/mL PBS) (ratio sulfanilamide/ovalbumin = 400/1). Next, 4 mL glutaraldehyde (0.5%) was added in drops to the mixture, whereafter the reaction mixture was stirred for 3 h at room temperature: sulfa-glut-ova (1).

Conjugation with glutaraldehyde according to Märtlbauer (1993). Briefly, 0.4 mmol sulfanilamide was diluted in 8 mL dimethylformamide and added to a solution of 0.003 mmol bsa or ova in 16 mL phosphate buffered saline (PBS, 0.15 M pH 7.4) (ratio

sulfanilamide/ovalbumin = 133/1). Next, 0.15 mL glutaraldehyde (25%) was added in drops to the reaction mixture and subsequently stirred for 3 h at room temperature: sulfa-glut-bsa (2) and sulfa-glut-ova (2).

All conjugates were dialyzed during 3 days against several changes of PBS before they were stored at –20°C.

In order to determine the amount of sulfonamide molecules bound to the carrier protein (molar incorporation), the protein concentrations were first determined with the BCA assay (Schmidt et al., 1985). Sulfonamides do not react with the BCA components. Next, the amount of bound sulfonamide in the conjugate was determined by measuring the absorbance at 280 nm. Because the carrier-protein and the bound sulfonamide in the conjugate both show absorbance at 280 nm, the absorbance of a sample containing only the carrier-protein at a concentration equal to the one in the conjugate, was also measured and substracted from the absorbance of the conjugate to obtain the absorbance of the sulfonamide. A calibration curve for the sulfonamide was established plotting the concentration of a standard dilution of the sulfonamide against the absorbances at 280 nm and was used to extrapolate the concentration of sulfonamide in the product, and consequently to calculate the amount of sulfonamide molecules per carrier molecule.

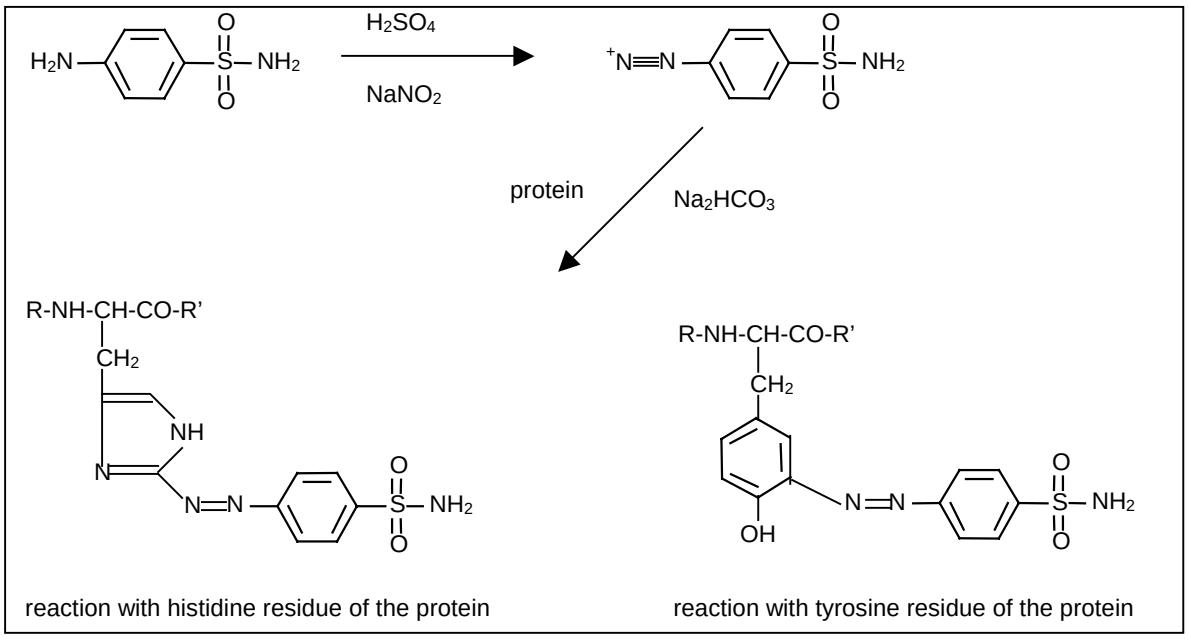


Figure 8.1: Conjugation of sulfanilamide to proteins by diazotation

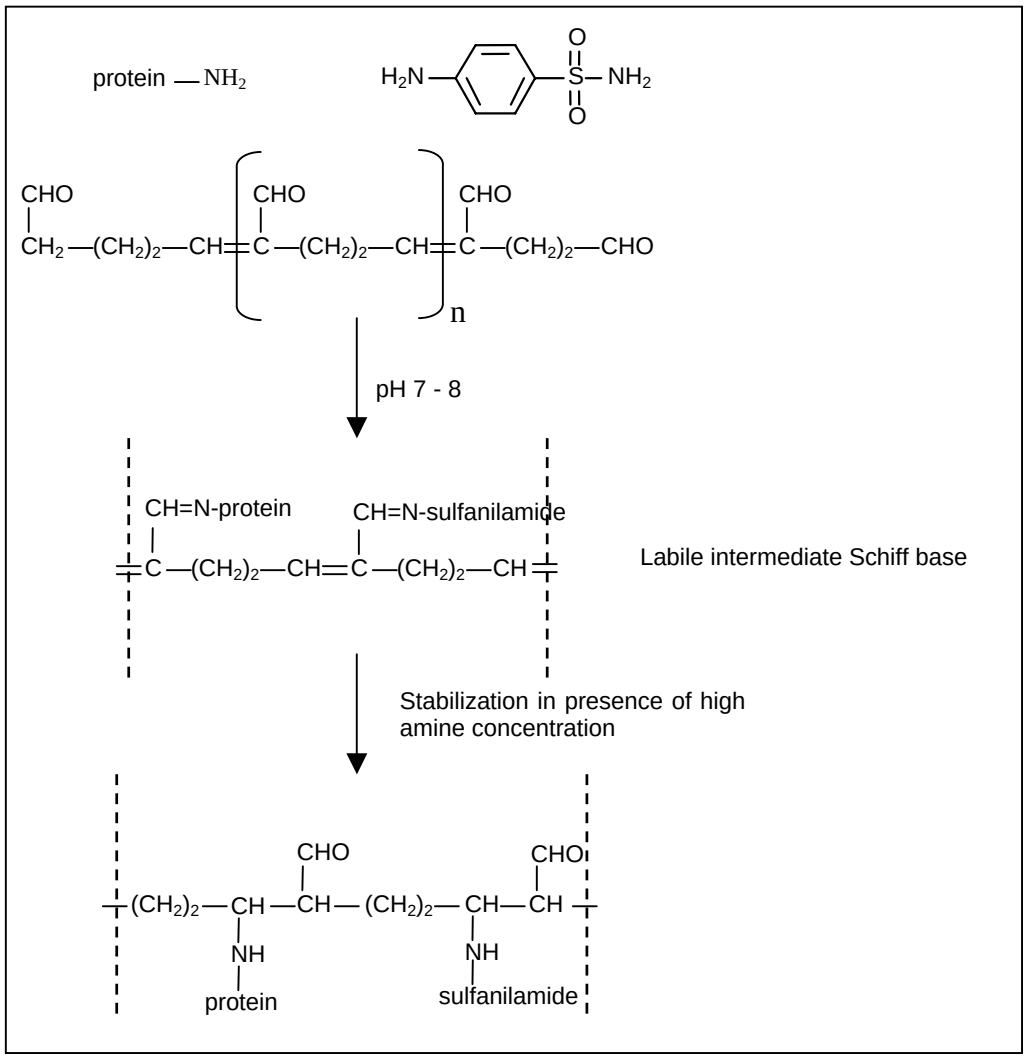


Figure 8.2: Conjugation of sulfanilamide to proteins using glutaraldehyde

Conjugation with a succinimide ester followed the method described by Kitagawa et al (1988) and van de Water (1990): a) Introduction of sulfhydryl groups on the carrier-protein (acetylthio-carrier-protein): 4.08 mmol s-acetylmercaptosuccinic anhydride (SAMSA) was added slowly to 0.077 mmol carrier-protein dissolved in 15 mL 0.1M potassium phosphate buffer pH 7.3 while maintaining the pH between 7 and 7.5. Once all SAMSA added, the pH was lowered to pH 6 by adding 1N HCl. The solution was dialyzed during 1 week against distilled H_2O whereafter the conjugate was lyophilized.

b) Removal of the acetyl group of acetylthio-carrier-protein: 10 µL deoxygenated 0.1M hydroxylamine was added to 20 mg lyophilized acetylthio-carrier-protein in 500 µL deoxygenated 0.1M phosphate buffer pH 7.3. The solution was then mixed under N_2 until no

further increase in number of sulfhydryl groups could be observed. The number of sulfhydryl groups was determined using the Ellman standard method (Ellman, 1959).

c) 3-maleimidobenzoic-n-hydroxysuccinimide ester (0.015 mmol MBS) in 0.5 mL tetrahydrofuran was added to 0.015 mmol sulfanilamide dissolved in 1 mL 0.05 M sodium phosphate buffer pH 7. The mixture was then incubated during 1 h while gently stirring. Subsequently, tetrahydrofuran was removed by mixing the solution under N_2 and the excess of MBS by extraction with 3 times 5 mL methyleenchloride/ether (1:2; vol/vol). The aqueous phase contained the MBS coupled sulfanilamide (sulfa-MBS) and was used in the next step.

d) The thio-carrier-protein solution was added to the sulfa-MBS solution and incubated for 2 h at 25 °C. The mixture was dialyzed against PBS during 3 days. Aliquots of the conjugates sulfa-MBS-ova and sulfa-MBS-bsa were stored at -20°C.

The molar incorporation was established by determining the number of free sulfhydryl groups left after coupling (Ellman, 1959) and substracting this amount from the number determined in step b. Since one sulfanilamide molecule only binds to one sulfhydryl group, the amount of bound sulfanilamide molecules equals the amount of reacted sulfhydryl groups: number of sulfanilamide molecules = $[(Ev-En)/Em]*f *N_A$

Ev = absorbance at 412 nm of the thio-carrier solution after reaction with Ellman's reagens

En = absorbance at 412 nm of the final product after reaction with Ellman's reagens

Em = molar extinction coefficient for the Ellman's reagens at 412 nm (13600)

N_A = number of Avogadro

f = dilution factor

The amount of carrier-protein molecules was determined by measuring the absorbance at 280 nm of the thio-carrier-protein solution before adding sulfa-MBS. The molar incorporation is expressed as the number of sulfanilamide molecules bound to one carrier molecule in the final product.

Conjugates with sulfonamide derivatives

The synthesis of N-sulfanyl-4-aminobenzoic acid (S) was described by Muldoon et al. (1999). For the coupling of S to bsa and ova, the aromatic amino group of S was protected with citraconic anhydride. Hereto, 20 mg of S was diluted in 1 mL DMSO whereafter 1 mL distilled water was added. The pH of this solution was adjusted to pH 8.5 using 1 M NaOH. Then, 2 mL citraconic anhydride solution (3.2 mg citraconic anhydride /mL distilled water)

was added in drops whereafter 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-p-toluenesulfonate (MEDC, 24.2 mg in 200 µL distilled water) was added to the mixture for 10 min at room temperature maintaining the pH at 8.5. Meanwhile, the carrier protein (39 mg bsa or 26 mg ova) was diluted in 2 mL distilled water and the pH was adjusted to pH 8.5. The diluted protein was then added to the reaction mixture and incubated for 2 h at room temperature. Citraconic anhydride was removed from the aromatic amino group of S by dialysis of the reaction mixture against 100 mM sodium acetate for 3 h at room temperature. Then the mixture was dialyzed against PBS. The conjugates S-ova and S-bsa were stored at –20 °C. The coupling efficiency was determined as done for the conjugations with glutaraldehyde.

The synthesis of the sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS) was described by Sheth and Sporns (1991). TS was coupled to klh, bsa and ova according to Haasnoot et al. (2000a). Briefly, TS (60 mg), N-hydroxysuccinimide (NHS, 35 mg) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC, 37.6 mg) were diluted in 1.5 mL DMF and incubated overnight at 4°C. The dicyclohexylurea precipitate was removed by centrifugation (12 000 g, 10 min) and 0.5 mL of the supernatant was added to 1mL of the cooled protein solution (5 mg/mL in PBS) whereafter the pH of the mixture was adjusted to 7.6. After stirring overnight at 4°C, the reaction mixture was dialyzed against 8M urea, then against 0.5M ammonium bicarbonate and finally against 0.25M ammonium bicarbonate (4l). The conjugates TS-ova, TS-bsa and TS-klh were stored at –20 °C. The coupling efficiency was determined as done for the conjugations with glutaraldehyde, except for the klh-conjugates.

N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS) was synthesized and conjugated to klh, ova and bsa as described (Assil et al., 1992; Haasnoot et al., 2000a).

Biotinylation of TS and PS

TS (1.8 mg) or PS (2.6 mg), N-hydroxysuccinimide (NHS, 1.25 mg) and MEDC (4.8 mg) were diluted in 300 µL DMSO and incubated overnight at room temperature while shacking. The mixture was added to a biotin-LC-PEO-amine solution (4.6 mg diluted in 400 µL PBS). After shacking overnight at room temperature, the reaction mixture was brought to 1 mL with PBS and aliquots of TS-bio and PS-bio were stored at –20°C. It was not necessary to separate the unreacted biotin from the biotin-labelled sulfonamide because no background signals were observed when using TS-bio or PS-bio in the antibody ciELISA.

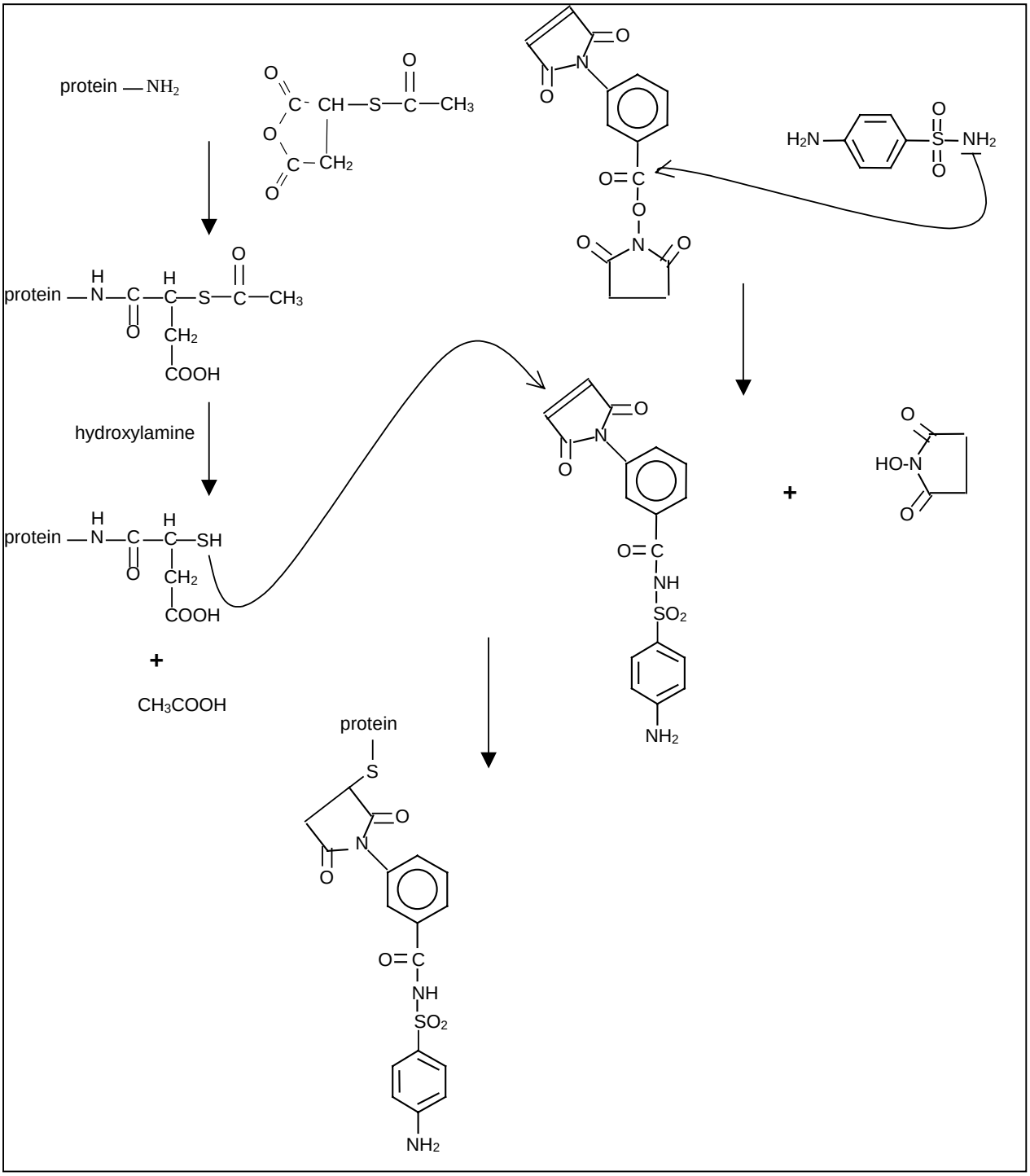


Figure 8.3: Conjugation of sulfanilamide to proteins using a succinimide ester coupling method

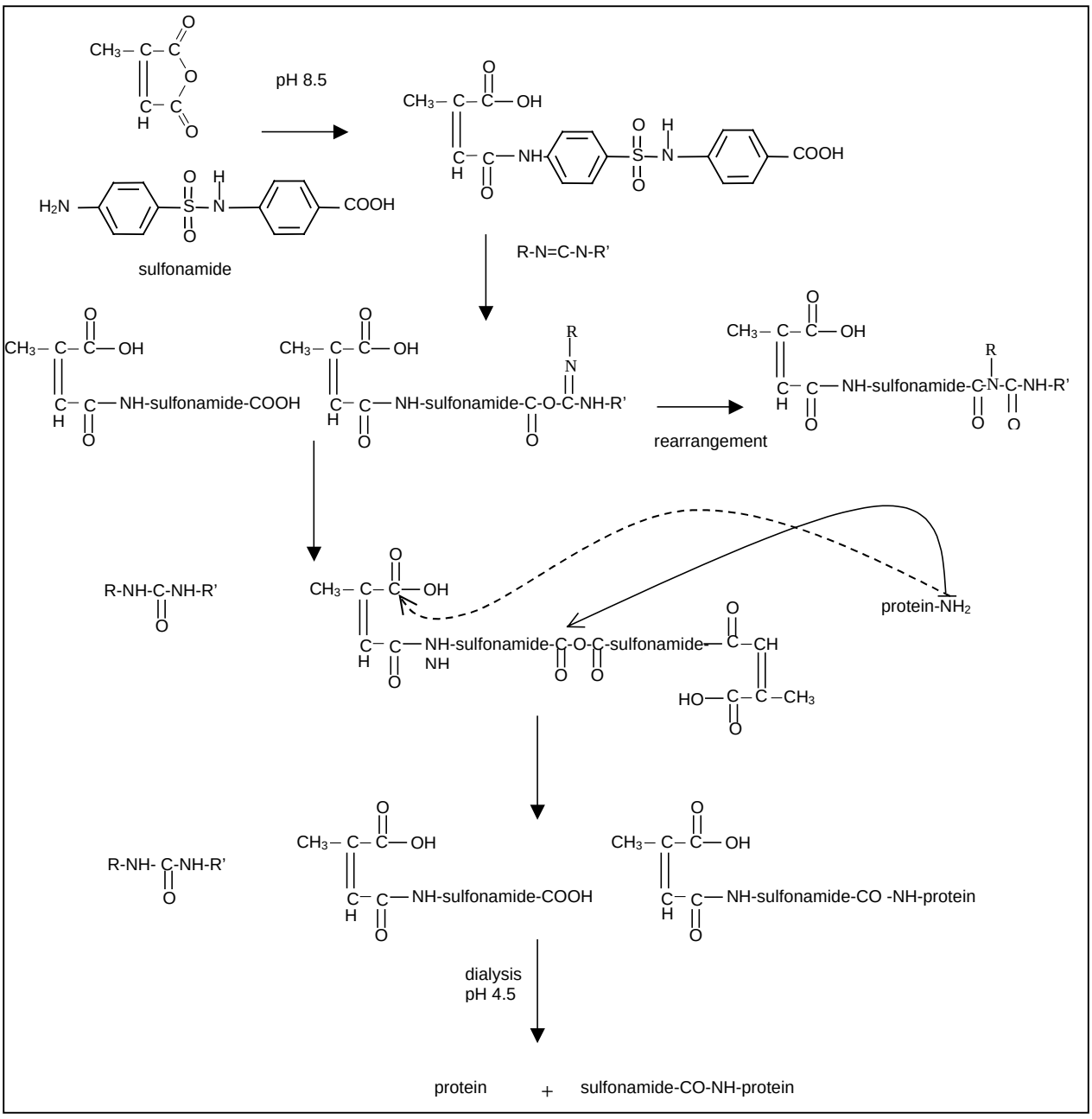


Figure 8.4: carbodiimide mediated conjugation method.

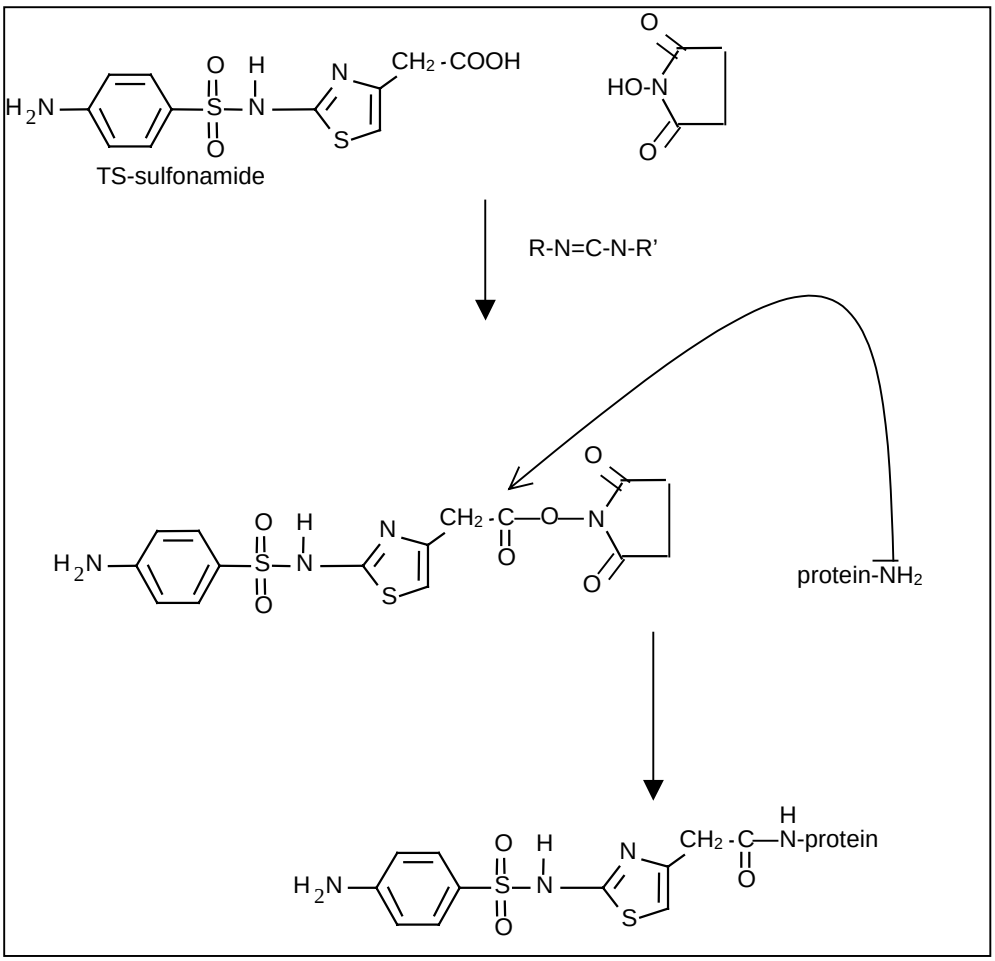


Figure 8.5: Carbodiimide mediated coupling method for the conjugation of TS-sulfonamide to proteins. The conjugation of PS-sulfonamide to protein was performed with the same procedure.

Immunization and production of mAb

Balb/c mice (minimum 10 weeks old) were immunized with an intraperitoneal injection of 50 µg of sulfonamide-protein conjugate (emulsified in 100 µL sterile PBS and 100 µL CFA), followed 4 weeks later by a second (and third) intraperitoneal injection with the same amount of conjugate (emulsified in sterile PBS and IFA). Blood samples were collected from the tail vein of the mice 2 weeks after each immunization and treated with kaolin as previously described *(*Van den Broeck et al., 1999*),* before analysis in ELISA for the presence of sulfonamide-specific antibodies. For the final booster injection, an intravenous immunization is preferred above an intraperitoneal one (Cliquet et al., 2001). But, to our experience, intravenous injection can sometimes fail, probably because it is a delicate manipulation and some of the solution is injected intradermal or subcutaneous. To overcome this risk, the final boost consisted of an intravenous (100 µg conjugate in sterile PBS) and an intraperitoneal injection (100 µg of conjugate emulsified in sterile PBS and IFA), and fusion

was performed 4 days later instead of three (for intravenous injection) or five (for intraperitoneal injections). In our hands, this procedure works excellent.

Hybridomas were obtained by polyethylene glycol mediated fusion of $SP_2OAG/14$ mouse myeloma cells with splenocytes from immunized mice. The isolation of lymphocytes, the culturing of the myeloma cells, the polyethylene glycol mediated fusion and the cloning of the hybridomas were performed according to the procedures previously described (Harlow and Lane, 1988; Cliquet et al., 2001). The supernatants of the hybridomas were tested twice in the screening ELISA coated with TS-ova. Hybridomas exhibiting absorbances higher then 2.0 were expanded to 24-well culture plates and tested for their specificity in the antigen ciELISA coated with PS-ova. The hybridomas with the highest specificity and sensitivity were cloned twice.

Screening ELISA

Microtiter plates were coated overnight at 4 °C with sulfonamide-protein conjugate (100 µL/well) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). The plates were washed 3 times with PBS containing 0.05% Tween®20 between each incubation step. Free binding sites were blocked with 200 µL of 5% glycine in coating buffer for 2 h at 37 °C. Subsequently, 100 µL/well of an appropriate dilution of sera or hybridoma supernatant in dilution buffer (PBS containing 3% bsa and 0.05% Tween®20), was added. The plates were incubated for 1 h at 37 °C. Then, 100 µL/well of rabbit anti-mouse immunoglobulins conjugated to peroxidase in dilution buffer was added for 1 h at 37 °C. Subsequently, 50 µL/well enzyme substrate ABTS solution was added. After incubation at 37 °C, the absorbance was measured at 405 nm.

Antigen-coated competitive inhibition ELISA (antigen ciELISA)

The only difference between the antigen ciELISA and the screening ELISA was that in the former, the samples (sera and hybridoma supernatant) were incubated in the wells of the coated plate with a sulfonamide mixture (10 µg/mL) containing sulfamethazine, sulfadiazine, sulfadimethoxine and sulfathiazole, or with serial dilutions (concentrations ranging from 10 to 0.001 µg/mL) of a sulfonamide.

The competition in the ELISA between a free sulfonamide in the sample and the coated sulfonamide was calculated with the formula: competition (%) = $(1-(A/A_o))*100$ with A= absorbance of a tested sample solution and A_o the absorbance of a similar solution without sulfonamide.

Antibody-coated competitive inhibition ELISA (antibody ciELISA)

Microtiter plates were coated overnight at 4 °C with rabbit anti-mouse immunoglobulins (0.1 µg/100 µL/well) diluted in bicarbonate coating buffer. Between each step, the plates were washed 3 times with PBS containing 0.05% Tween®20. Free binding sites were blocked with 5% Tween®80 in coating buffer for 2 h at 37 °C. Then, 100 µL/well of an appropriate dilution of the monoclonal antibody in PBS (or only PBS for background measurements) was added for 1 h at 37 °C. Subsequently, sulfonamides in dilution buffer were added and incubated for 1 h at 37°C. Without washing the plates, a biotinylated sulfonamide in dilution buffer was added for 30 min at 37 °C. After washing 100 µL/well of streptavidine-peroxidase in dilution buffer was added for 30 min at 37 °C. Finally, the plates were washed again whereafter TMB solution (100 µL/well) was added. The absorbance was measured at 650 nm.

Results

Sulfonamide immunogens and antibody response

Different sulfonamide-protein conjugates were used for immunization (Table 8.1). Smt-bgg and azocasein were commercially available. Sulfanilamide was coupled to albumins using glutaraldehyde or a succinimide ester as cross-linker, and using a diazotation reaction. Three sulfonamide derivatives were synthesized containing a carboxyl group in their side chain (PS, TS and S; Figure 8.6) and were linked to proteins using a carbodiimide coupling method. In these conjugates the common structure of sulfonamides was left unchanged, so that they could induce group-specific antibodies.

Blood samples were collected two weeks after each immunization and were tested in ELISA for the presence of anti-sulfonamide antibodies (Tables 8.2 and 8.3).

No sulfanilamide-specific antibodies were induced after immunization with the glutaraldehyde (sulfa-glut-albumin (1) and (2)) or succinimide (sulfa-MBS-albumin) conjugates. Sera of mice immunized with sulfa-bsa showed high absorbances in the ELISA coated with azocasein (Tables 8.2 and 8.3). However, the binding of these antibodies to azocasein could only be slightly inhibited by adding sulfonamides (Table 8.2). This was independent of the number of immunizations. Similarly, high absorbances were also detected for blood samples collected after immunizations with azocasein and analyzed in the ELISA coated with sulfa-bsa, and again only a slight inhibition was observed in the presence of

sulfonamides (Table 8.2). So again competition average remained low, independent of the number of immunizations.

Table 8.1: Methods used for sulfonamide-protein conjugation

Sulfonamide-protein conjugate	Coupling method	Hapten/protein ratio
sulfa-glut-ova(1)	Glutaraldehyde, method Van Regenmortel	165/1
sulfa-glut-bsa(2), sulfa-glut-ova(2)	Glutaraldehyde, method Martlbauer	ND[1]
sulfa-bsa	Diazotation	ND
azocaseïn, smt-bgg	Commercial conjugates	No data available
sulfa-MBS-bsa	Succinimide ester	10/1
sulfa-MBS-ova	Succinimide ester	ND
S-ova, S-bsa	Carbodiimide	3/1 – 8/1
PS-ova, PS-bsa, PS-klh	Carbodiimide	ND
TS-ova	Carbodiimide	37/1
TS-klh	Carbodiimide	ND

[1]ND = not determined

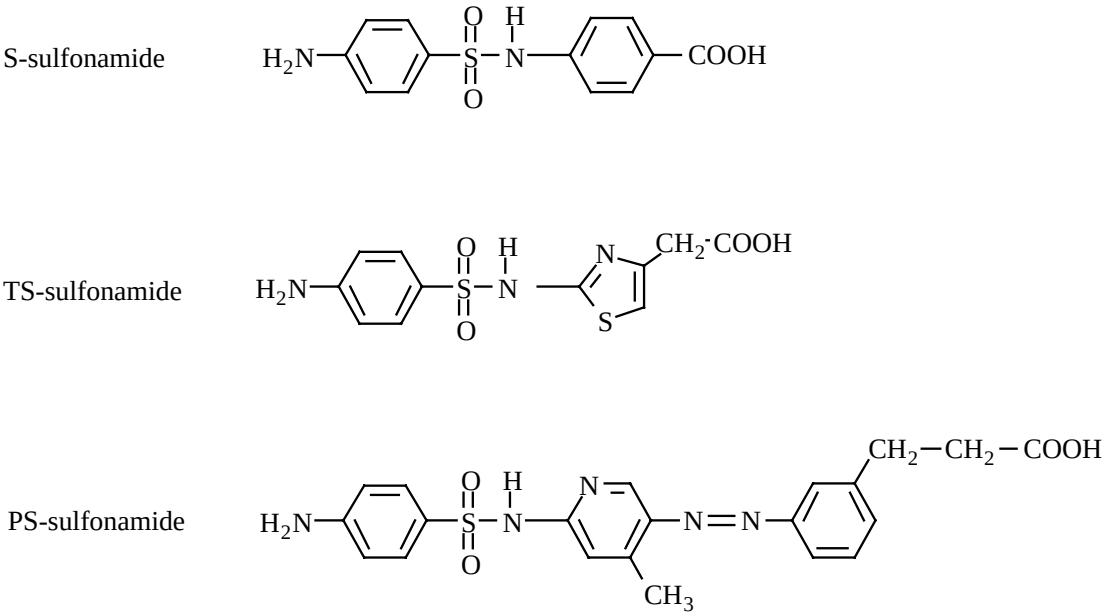


Figure 8.6: Structures of the sulfonamide derivatives. S-sulfonamide = N-sulfanyl-4-aminobenzoic acid; PS-sulfonamide = N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide; TS-sulfonamide = (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide.

Table 8.2: Immunogenicity of different sulfonamide-protein conjugates

Immunogen	Number of mice	Number of injections	Antigen ciELISA				
			Dilution serum	Coating antigen	Absorbance[1] Average (SD)	Competitor 10 µg/mL	Competition[2] average (SD) %
Sulfa-glut-bsa(1)	2	4	100	Sulfa-MBS-ova	-		
Sulfa-glut-ova(2)	2	4	100	Sulfa-MBS-bsa	-		
Sulfa-MBS-bsa	2	4	100	Sulfa-glut-ova(1)	-		
Sulfa-bsa	2	9	1000	Azocaseïn	+++ (+)	Smix[3]	< 20%
Azocaseïn	4	7	1000	Sulfa-bsa	+++ (++)	Smix	< 20%
Smt-bgg	3	7	1000	S-albumin	+ (+)	Sulfanilamide	< 20%
			1000	TS-ova[4]	++++	Smix	80 %
S-albumin	12	4	1000	S-albumin[5]	++++ (+)	Smix	< 20%
TS-klh	6	3 (2)	10000	TS-ova	+++ (++)	Smix	79 (10)
			10000	PS-ova	+ (+)	Smix	44 (36)
TS- & PS-klh	4	4	10000	TS-ova	++ (++)	Smix	65 (12)
			10000	PS-ova	++ (++)	Smix	60 (32)

[1]Scores are given for the average of the absorbance obtained in the antigen ciELISA for the sera of a number of mice in absence of competitor: - = < 0.200; + = 0.200 – 0.500; ++ = 0.500 – 1.000; +++ = 1.000 – 1.800; ++++ = > 1.800.
[2]Competition (%) = [100 – (absorbance in presence of competitor/ absorbance in absence of competitor)] x 100
[3]Smix = mixture of four sulfonamides (sulfamethazine, -diazine, -dimethoxine and –thiazole) at a final concentration of 10 µg/mL
[4]Sera of the best responding mouse
[5]The albumin of the coating antigen was different from the one in the immunogen.

Three mice were immunized with smt-bgg. The sera of the best responding mouse reacted with azocasein, sulfa-bsa, S-ova and TS-ova (Table 8.3), and showed 80% inhibition in the presence of 10 µg/mL of a mix of sulfonamides in the ciELISA coated with TS-ova (Table 8.2). This means that the antibodies recognized a common structure. Unfortunately, this mouse died. The antibody titers of the other mice did not became high enough, even after seven immunizations.

Immunizations with the S-conjugates led to high antibody responses, highly specific for the S-conjugates (Table 8.3). However, the free S-molecule was only weakly recognized (data not shown) as were the 4 sulfonamides in the competitor mixture (at a concentration of 10 ppm, less than 20% inhibition was observed, table 8.2). Fusion experiments with the splenocytes of mice immunized with these conjugates did not result in any group-specific mAb.

High antibody responses were also obtained after immunizations with the PS- and TS-klh conjugate. Sera tested in the antigen ciELISA had titers from 100 000 to 300 000 on TS-

ova coating (Table 8.3). Furthermore, these antibodies could be inhibited in the ELISA with different sulfonamides (Table 8.4). Lower titers (20 000 tot 50 000) were obtained on PS-ova coating (Table 8.2). No response was measured in the ELISA coated with azocasein, sulfa-bsa or S-ova (Table 8.3).

Production of mAb

Three mice (m90, m98 and m95, table 8.4) differently immunized with TS- and PS-conjugates were selected for fusion experiments. In table 8.4 the cross-reactivities are given for different sulfonamides detected in the antigen ciELISA with sera of the three mice. The three sera had the highest affinity for sulfachloropyridazine and sulfathiazole, a lower affinity for sulfadiazine, -dimethoxine and –pyridine, and the lowest affinity for sulfamethazine, -merazine and sulfisoxazole. The sensitivity of the three sera for sulfonamides was higher in the ELISA coated with PS-ova as compared to TS-ova.

Table 8.3: Reactivity of sera on different coating antigens in a screening ELISA. The results of the sera obtained from the best responding mouse of each group immunized with different immunogens, are presented.

	Reactivity[1] of sera on different coating antigens					
Immunogen	Azocasein	Sulfa-bsa	S-ova	TS-ova	ova	bsa
Smt-bgg	++	+++	++	++++	-	-
Azocaseïn	++++	++++	-	-	-	-
Sulfa-bsa	+++	++++	-	-	-	-
S-bsa	+	+	++++	-	-	+
TS-&PS-klh	-	-	-	++++	-	-
TS-klh	-	-	-	++++	-	-

[1]Scores are given for the absorbance obtained in the screening ELISA for 1/1000 diluted sera: - = < 0.200; + = 0.200 – 0.500; ++ = 0.500 – 1.000; +++ = 1.000 – 1.800; ++++ = > 1.800.

The antigen ciELISA

The cross-reactivities of the mAb for sulfonamides were determined in the antigen ciELISA coated with PS-ova (Table 8.5). The antibodies could be divided into three groups. The mAb of group I had a very high affinity for sulfathiazole, -methoxazole and –chloropyridazine, but also recognized sulfadiazine, -dimethoxine and –pyridine at an acceptable level. The antibodies were at least ten times less reactive to sulfamethazine and –merazine. The antibodies of group II had the same reactivities as group I, except that the sensitivity for sulfamethazine and –merazine was at least four times higher. The mAb also recognized sulfadiazine at least four times better than group I. Group III showed better

recognition of sulfamethazine and –merazine as compared to –diazine and -dimethoxine. But the recognition of both latter was at least four times lower as for group II. The antibodies seemed to recognize sulfamethazine and –merazine in the same way. However, mAb 27G3 (Group IV, Haasnoot et al., 2000a) obtained after TS-klh immunization, had higher affinity for sulfamerazine as compared to –methazine.

Cross-reactivities of mAb 3B5B10E3 were determined for structural related molecules and drugs currently administered together with sulfonamides: diuretics (Furosemide, acetazolamide, hydrochlorothiazide, bumetanide), thiamphenicol, florphenicol, lidocaine and para-aminobenzoic acid (PS-ciELISA). The diuretics only have the $–SO_2NH-$ group in common with sulfonamides. Thiamphenicol and florphenicol have a $–SO_2-$ group, lidocaine has nothing structural in common with sulfonamides. Para-aminobenzoic acid contains the common sulfonamide *p*-aminobenzoyl ring. The monoclonal recognized all four diuretics (IC50 > 10 µg/ml), but none of the other molecules. This indicates that the epitope recognized by the monoclonal contains the $–SO_2NH-$ group. Substitution of this sulfonamide-group on a benzene ring favors the binding of the monoclonal since sulfonamides are recognized with higher sensitivity (IC50 < 1 µg/ml; table 8.6).

Table 8.4: Cross-reactivities[1] (IC50, ng/mL) for different sulfonamides of three mice sera (m90, m95 and m98) in the antigen ciELISA (coated with PS-ova)

Mouse	m90		m95		m98	
Immunogen	TS-klh (2x), PS-bsa (2x)[2]		TS-klh (2x), PS-klh (1x)[2]		TS-klh (3x)	
Coating	PS-ova	TS-ova	PS-ova	TS-ova	PS-ova	TS-ova
Dilution serum	1/5000	1/20000	1/8000	1/20000	1/8000	1/20000
Sulfonamide (sulfa-)	IC50 (ng/mL)					
-chloropyridazine	7	100	30	400	30	600
-thiazol	7	85	30	150	3	100
-diazine	70	600	500	800	300	7000
-dimethoxine	400	3000	500	3000	600	1500
-pyridine	150	900	800	4000	1000	4000
-methazine	1000	>10000	10000	>10000	10000	>10000
-merazine	600	>10000	4000	>10000	10000	>10000
sulfisoxazole	3000	3000	10000	4000	>10000	>10000

[1]the cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen ciELISA.
[2]alternated injected with TS-klh and PS-bsa or PS-klh

Table 8.5: Cross-reactivities[1] (IC50, ng/mL) for different sulfonamides of different monoclonal antibodies in the antigen ciELISA (coated with PS-ova)

GROUP	I					II		III	IV
Mouse	m95	m95	m98	m98	m98	m98	m90	m95	Haasnoot[2]
Monoclonal antibody	6H12H3	18E1D9	17D11E6	11F5D5B12	1C12B11G5	3A10E3	3B5B10E3	14D6D6 14D6C9	27G3A9B10
sulfathiazole	20	7	5	10	15	< 20	30	350	10
sulfamethoxazole	20	7	9	10	15	< 20	9	1000	150
sulfachloropyridazine	30	10	15	40	30	< 20	< 20	600	4
sulfadiazine	1000	300	250	500	1000	60	30	>10 000	80
sulfadimethoxine	1000	150	200	500	1000	200	100	>10 000	250
sulfapyridine	1000	300	300	1000	2000	400	350	3000	30
sulfamethazine	>10 000	4000	8000	>10 000	>10 000	1050	1050	4000	8000
sulfamerazine	>10 000	4000	8000	>10 000	>10 000	700	600	4000	500
sulfisoxazole	>10 000	10 000	6000	>10 000	>10 000	400	350	>10 000	250

[1]The cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen ciELISA.
[2]Haasnoot et al. (2000a)

Table 8.6: Cross-reactivity[1] (IC50, ng/mL) and limit of detection[2] (LOD, ng/mL) for different sulfonamides in buffer solution detected with mAb 3B5B10E3 in the antigen ciELISA (coated with PS-ova) and in the antibody ciELISA.

	Antigen ciELISA		Antibody ciELISA	
	LOD (ng/mL)	IC50 (ng/mL)	LOD (ng/mL)	IC50 (ng/mL)
sulfathiazole	5	30	3	50
sulfamethoxazole	< 1	9	< 1	10
sulfachloropyridazine	< 1	< 20	ND	ND
sulfadiazine	6	30	3	50
sulfadimethoxine	50	100	30	500
sulfapyridine	80	350	70	1050
sulfamethazine	300	1050	400	4000
sulfamerazine	200	600	ND	ND
sulfisoxazole	80	350	200	2000

[1]The cross-reactivities = concentration of a sulfonamide (ng/mL) required to obtain 50% inhibition (IC50) in the antigen ciELISA.
[2]Limit of detection = concentration read from the calibration curve at a response (absorbance) minus 3 standard deviation from the mean (n=12) for the zero standard (buffer sample without sulfonamides)

The antibody ciELISA

Monoclonal antibody 3B5B10E3 of group II was selected to develop an antibody ciELISA. Therefore, the mAb was captured on an ELISA-plate precoated with mouse-specific polyclonal antibodies. Furthermore, competition of sample sulfonamide for binding to the mAb was done using TS-biotin because with PS-biotin, no or very little absorbances were obtained. The cross-reactivity values (IC50) and limit of detection (LOD, Babiel et al., 1993) were determined for different sulfonamides in buffer solution analyzed in the antigen ciELISA and in the antibody ciELISA (Table 8.6). Most of the sulfonamides were recognized with the same sensitivity (LOD) in both ELISAs.

Discussion

The aim of this study was the production of mAb specific for all sulfonamides. With such antibodies, a sensitive, group-specific immunoassay could be developed for the detection of sulfonamides in food products.

Our first approach was the use of sulfanilamide as hapten since it is the common structure of all sulfonamides and it does not have a “disturbing” side group. Antibodies against sulfanilamide should be group-specific. Different coupling methods were applied to

link sulfanilamide to proteins using glutaraldehyde (sulfa-glut-albumin (1) and (2)) or a succinimide (sulfa-MBS-albumin) as cross-linker or by diazotation (sulfa-bsa and azocasein). No immune response was obtained after immunization with the sulfa-glut-albumin or sulfa-MBS-albumin. For the glutaraldehyde coupling with the procedure of Van Regenmortel (1988), a sulfa-glut-ova (1) conjugate was developed with a molar incorporation of 165/1 (Table 8.1). The reaction of glutaradehyde with proteins involves mainly lysine residues, as well as the α-amino group and sulfhydryl group of cysteine residues of the protein. Ovalbumin has 20 lysine and 4 cysteine residues accessible for glutaraldehyde conjugation and therefore the highest molar incorporation possible is 24/1, if a one to one ratio is respected. However, glutaraldehyde can form polymers (Van Regenmortel, 1988; Hermanson, 1996). This property in combination with the high ratio of sulfanilamide / ovalbumin (400/1) mixture used for coupling could explain the high coupling ratio of 165/1. The absence of immune response using this sulfa-glut-ova (1) conjugate could be due to the high load of hapten on the carrier protein. Ideally, a hapten/protein ratio of 5-20 is needed to obtain a good immunogen (Van Regenmortel, 1988). Therefore the glutaraldehyde procedure according to Märtlbauer (*20*) was applied (sulfa-glut-albumin (2)). Märtlbauer (1993) reported sulfonamide conjugates using this method with a molar incorporation of 7/1 and high antibody titers after immunization. However, our conjugates were still not immunogenic. Unfortunately, the hapten/protein ratio was not determined. Haasnoot et al. (2000b) also did not determine the molar incorporation ratio and obtained only low titers after immunization with several sulfonamide-protein conjugates linked by glutaraldehyde. In previous work (Cliquet et al., 2001), we immunized mice with ampicillin coupled to albumins using the glutaraldehyde procedure according to Märtlbauer (coupling efficiency of 8 to 16) and also found the conjugates to be weak immunogens.

The molar incorporation of our sulfa-MBS-albumin conjugates (10/1, table 8.1) was in accordance with other studies (Kitagawa et al., 1988; van de Water, 1990; Cliquet et al., 2001). In previous work we constructed ampicillin-MBS-albumin conjugates with a hapten/protein ratio of 8/1 to 13/1 but obtained only a moderate immune response (Cliquet et al., 2001).

High antibody titers were obtained after immunization with the diazotation conjugates sulfa-bsa and azocasein. However, these antibodies could only be slightly inhibited in ELISA by free sulfonamides or even sulfanilamide. This means that the antibodies were highly specific for the bound sulfanilamide molecule. The diazotation reaction was used by others

for the development of antibodies specific for sulfamethazine (Fleeker and Lovett, 1985), sulfamerazine (Garden and Sporns, 1994) and sulfathiazole (Sheth et al., 1990) leading in all cases to antibodies able to recognize the respectively free sulfonamide molecule. Sulfanilamide does not have a side chain like sulfamethazine, -merazine and –thiazole and this could be the reason why only low amounts of sulfanilamide-specific antibodies were obtained. The available epitope in the sulfanilamide conjugates is probably build up by the linkage at the aromatic amino group (N4) and the benzene ring (Figure 3.1, chapter 3).

Our second approach was the use of sulfonamide-protein conjugates in which the sulfonamide is linked at its side chain, leaving the common group unchanged. In the commercially available smt-bgg conjugate sulfamethazine was linked through its pyrimidin ring to bovine gamma globulin (bgg). Polyclonal antibodies obtained after immunization with this antigen are also commercially available. These antibodies recognized several sulfonamides and therefore the antigen was believed to induce group-specific antibodies. Especially one of the three mice immunized with this smt-bgg indeed produced sulfonamide-specific antibodies. Unfortunately, no fusion experiments could be performed with the splenocytes of this mouse.

Immunizations with the N-sulfanilyl-4-aminobenzoic acid-protein conjugates (S-conjugates) led to a large production of antibodies (high absorbances), but all of them were highly specific for the conjugates. Fusion experiments with the splenocytes of mice immunized with the S-albumin conjugates did not result in any group-specific antibody. Muldoon et al. (1999) used the same sulfonamide derivative coupled to klh. Only one of five immunized mice produced high titers of antibodies able to recognize other sulfonamides. Fusion experiment resulted in only one monoclonal antibody specific for the group of sulfonamides.

Using the sulfonamide derivatives of Sheth and Sporns (1991) and Assil et al. (1992) was more successful. Haasnoot et al. (2000a; 2000b) also used the same approach and found out that immunization of mice with PS-klh led to the recognition of sulfonamides (sulfamethazine, -merazine, -diazine, -dimethoxine) that were not well recognized after TS-klh immunization. Consequently, we tried to guide the immune response in the direction of recognition of the common sulfonamide structure by immunizing the animals alternately with TS- and PS-protein conjugates. All mice immunized with TS-klh showed high titers in the

ELISA with TS-ova coating. As expected from the results of Haasnoot et al. (2000a; 2000b), immunization with PS-bsa or PS-klh did not induce a high increase of the titer. However, the mouse only immunized with TS-klh had a lower antibody binding to sulfonamides containing a pyrimidinyl group (sulfamethazine, -merazine, -diazine) or a pyridinyl group (sulfapyridine) than the alternately immunized mice (Table 8.4). The detection of the sulfonamides was improved when the ELISA was coated with PS-ova as compared to TS-ova.

In contrast to the group-specific serum antibodies of the mouse immunized with smt-bgg, serum of the mouse immunized with TS-klh did not bind to coated azocasein, sulfa-bsa or S-ova (Table 8.3). Probably, the major epitope recognized by the TS-klh antibodies is more positioned toward the side chain of the sulfonamides, and therefore binding of these antibodies to the conjugated sulfanilamide or S-molecule could be sterically inhibited by the carrier protein. The major epitope recognized by the smt-bgg antibodies is probably more located at the side of the common sulfonamide structure and antibody binding is therefore less influenced by the side chain.

The mAb obtained after fusion experiments with the splenocytes of mice immunized with TS-klh could be divided, based on their cross-reactivities, in three groups, independently from which mouse they were deduced. This means that alternately immunization with two different immunogens will improve the broad specificity of the polyclonal serum but not of the individual mAb. Consequently, to obtain broad specific mAb, it would be sufficient to immunize with TS-klh, and to screen the hybridomas in ELISA coated with PS-ova.

Two ELISA-systems, an antigen-coated and an antibody-coated ciELISA, were constructed with mAb 3B5B10E3. Almost all tested sulfonamides were detected with the same sensitivity in both ELISA. Because the sensitivity of the antigen ciELISA was improved when PS-ova was used as coating antigen compared to TS-ova, the sensitivity of the antibody ciELISA would probably be improved using the biotinylated PS-sulfonamide PS-bio instead of TS-bio. However, very little absorbances were obtained with PS-bio.

Most sulfonamides, except sulfamethazine and sulfamerazine, can be detected in buffer solution at the MRL (100 ppb) in the antigen ciELISA with mAb 3B5B10E3. On the other hand, a lot of sulfamethazine-specific immunoassays already exist. Most of the time, such assays cross-react with sulfamerazine. Due to the diversity of the sulfonamide side chain, a broad specific immunoassay for sulfonamides using one monoclonal antibody is

probably not possible. Most likely, two or three immunoassays, each with a different monoclonal, have to be used for the screening of food products (Muldoon et al., 2000; Haasnoot et al., 2000a; Haasnoot et al., 2000c, Haasnoot et al., 2003). Haasnoot and coworkers (2000c) could detect sixteen sulfonamides in the BIAcore 2000 biosensor using a mixture of three group-specific monoclonal antibodies. However, a group-specific sulfonamide ELISA based on the use of several monoclonals has not been described yet. In further work, the detection of sulfonamides in meat samples using the antigen ciELISA with mAb 3B5B10E3 will be investigated.

Acknowledgements: This study is supported by the Ministry of Agriculture, Brussels, Belgium and by the Institute for Veterinary Inspection (IVK). The technical assistance of Els De Vogelaere was greatly appreciated. We thank Roeland Mijngheer for the synthesis of some of the sulfonamide derivatives and conclusions.

Chapter 9

Extraction procedure for sulfachloropyridazine in porcine tissues and detection in a sulfonamide-specific ELISA

Based on: P. Cliquet, E. Cox, W. Haasnoot, E. Schacht and B. M. Goddeeris. Extraction procedure for sulfachloropyridazine in porcine tissues and detection in a sulfonamide-specific ELISA. Analytica Chimica Acta, 2003, 494, 21-28.

Abstract

Sulfonamide-specific polyclonal rabbit antibodies were obtained after immunization with a sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide = TS) coupled to keyhole limpet hemocyanin. Using these antibodies, two sulfonamide-specific ELISAs were developed differing in coating antigen: TS-ovalbumin (TS-ova) and PS-ovalbumin (PS-ova, PS = N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide). The detection of sulfamethazine, sulfamerazine, sulfadimethoxine, sulfadiazine, sulfathiazole, sulfapyridine, sulfachloropyridazine and sulfisoxazole in buffer was analysed. Higher antibody titers were obtained in the ELISA coated with TS-ova (TS-ciELISA) as compared to the ELISA coated with PS-ova (PS-ciELISA), but the detection of sulfonamides was more sensitive in the PS-ciELISA, allowing the detection of all tested sulfonamides at the MRL-value (100 ng/ml).

In a subsequent step, an extraction procedure was developed for the detection of sulfonamides, in muscles, kidney, liver and fat, by both ELISAs using sulfachloropyridazine as model. As extraction buffer a carbonate/bicarbonate buffer (pH 10) was chosen in which sulfonamides are highly soluble. Differences in homogenizing techniques (high-speed mixer (ultraturax) versus vortex) and the effect of kaolin (hydrated aluminum silicate) treatment, to diminish the background signal in ELISA, were evaluated. The best extraction procedure was the one using a vortex mixer as homogenizer and no kaolin treatment. Sulfachloropyridazine was easily detected at the MRL in all tissues.

The decision limit and detection capability for sulfachloropyridazine in porcine kidneys were determined ($CC\alpha$ = 9.4 ppb, $CC\beta$ = 12.9 ppb). Good correlations were found between the PS-ciELISA and LC-MS/MS for the analysis of incurred porcine liver and kidney samples (r^2 = 0.88 and 0.77, respectively).

Keywords: Sulfonamide – ELISA – extraction procedure - porcine tissues

Introduction

Sulfonamides are chemotherapeutics widely used in veterinary medicine for the treatment of bacterial infections, and as feed additive (Long et al., 1990). As a result, sulfonamides can occur in food products from animal origin (Franco et al., 1990). To protect consumers from risks related to drug residues, maximum residue levels (MRL) have been established by law. In Europe, Canada and the United States, the MRL for the total amount of sulfonamides in edible tissues is 100 µg/kg (Anonymous, 1991; Anonymous, 1999). In Japan, this MRL is 20 µg/kg. Detection methods for sulfonamides include bioassays, thin-layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC, LC-MS/MS). Liquid chromatography is a sensitive and specific assay but is also very laborious and expensive. The method is very suitable for confirmation but not for screening of large amounts of samples. A rapid, sensitive and specific assay is needed to pick up positive samples in routine analyses, which then can be confirmed for the presence of sulfonamides by liquid chromatography. Therefore, during the past ten years, a variety of immunoassays were developed, each highly specific for an individual sulfonamide (Fleeker and Lovett, 1985; Dixon-Holland and Katz, 1988; Sheth and Sporns, 1990; Garden and Sporns, 1994; Muldoon et al., 2000a; Lee et al., 2001; Spinks et al., 2001). However, it would be more efficient to have one immunoassay able to detect all sulfonamides instead of an immunoassay for each individual sulfonamide (Sheth and Sporns, 1991; Assil et al., 1992; Muldoon et al., 1999; Spinks et al., 1999; Haasnoot et al., 2000a; Haasnoot et al., 2000b; Li et al., 2000).

The sulfonamides share a common *p*-aminobenzoyl ring moiety with an aromatic amino group at the N4-position and differ in the substitution at the N1-position. For the group-specific detection of sulfonamides, sulfonamide-specific competitive ELISAs were developed with polyclonal antibodies against the aromatic amino group. In the present study, the detection of different sulfonamides in buffer solution using these ELISAs and the development of an appropriate extraction procedure for the detection of sulfachloropyridazine in porcine tissues is described. The aim of our study was to develop a simple procedure that can be applied in further work for the extraction of all sulfonamides in porcine kidney as well as liver, muscle and fat. For homogenizing sample and extraction buffer the use of a rigorous method (ultraturax high speed mixer) was compared to a simple shaking method (vortex mixer). In previous experiments in which an extraction procedure for the detection of penicillins in meat samples was developed, kaolin treatment was found to be very efficient to

diminish background signals in ELISA (personal communication). In the present study, it was assessed if such a treatment was needed for the analysis in the sulfonamide-specific ELISAs.

Material and methods

Reagents

Sulfathiazole, sulfadiazine, sulfamethazine, sulfadimethoxine, sulfapyridine, sulfamerazine, sulfisoxazole, sulfachloropyridazine, para-aminobenzoic acid, bovine serum albumin (bsa), ovalbumin (ova), kaolin (hydrated aluminum silicate) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Bornem, Belgium). Furosemide, acetazolamide, hydrochlorothiazide, bumetanide, thiamphenicol, florphenicol and lidocaine were a kindly gift of the Department of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Ghent University. The TMB substrate solution was prepared by adding 3.3 mg TMB in 250 µl DMSO to 25 ml phosphate-citrate buffer (0.1M citric acid + 0.2M Na_2HPO_4; pH 4.3) containing 3.25 µl of a 30 % H_2O_2 solution. Dimethylsulfoxide (DMSO) was from VWR (Leuven, Belgium). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were provided by Difco Laboratories, Biotrading (Bierbeek, Belgium). Tween®20 (polyoxyethylene sorbitan monolaurate) was purchased from Merck-Belgolabo (Overijse, Belgium). Swine anti-rabbit immunoglobulins (code n°P0217) conjugated to peroxidase were obtained from DAKO Diagnostica (Prosan, Ghent, Belgium). ELISA microtiter plates (maxisorp®) were from NUNC (Life technologies, Merelbeke, Belgium). All other chemicals were of reagent grade.

Synthesis of sulfonamide-protein conjugates

N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS) was synthesized at the State Institute for Quality Control of Agricultural Products (RIKILT, Wageningen, Netherlands) and conjugated to ovalbumin (PS-ova) as previously described (Assil et al., 1992; Haasnoot et al., 2000a).

The sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS) was synthesized in the Laboratory of Organic Chemistry (Faculty of Sciences, Ghent University) as described (Sheth and Sporns, 1991). The TS-molecule was coupled to the

proteins keyhole lympet hemocyanin (klh) and ovalbumin (ova) according to Haasnoot et al. (2000a), resulting in the conjugates TS-klh and TS-ova.

Production of polyclonal antibodies

New Zealand white rabbits were immunized subcutaneously with 100 µg of the TS-klh conjugate. For the first immunization, the conjugate was emulsified in 500 µl sterile saline and 500 µl complete Freund's adjuvant. For all subsequent immunizations, at six weeks interval, incomplete Freund's adjuvant was used. Blood was sampled two weeks after each immunization. The serum was collected (3000 g, 20 min) and treated with kaolin (hydrated aluminum silicate) (Van den Broeck et al., 1999). Therefore one part of serum was mixed with four parts of kaolin and incubated for 30 min at room temperature. The mixture was centrifuged (3000 g, 20 min) and the supernatant was stored frozen. The antibodies pAb K3 were analysed in the competitive inhibition ELISA.

Competitive inhibition ELISA coated with TS-ova (TS-ciELISA) or with PS-ova (PS-ciELISA)

Microtiter plates were coated overnight at 4 °C with PS-ova or TS-ova (1 µg/ml; 100 µl/well) diluted in coating buffer (bicarbonate buffer, 0.05 M, pH 9.4). Between each incubation step, the plates were washed 3 times with phosphate buffered saline (PBS, 0.15 M pH 7.4) containing 0.05% Tween® 20. Free binding sites were blocked with 200 µl of 5 % glycine in coating buffer for 2 hours at 37 °C. Subsequently, 100 µl of the samples (tissue extract or standard dilution of sulfachloropyridazine in PBS) together with 100 µl of an appropriate dilution of pAb K3 in PBS containing 3% BSA and 0.05% Tween® 20, were added to the wells (specific absorbance). For background measurement, the samples were added without pAb K3 (background absorbance). The plates were incubated for 1 hour at 37 °C. Then, 100 µl of the peroxidase labelled swine anti-rabbit immunoglobulins (diluted in PBS containing 0.05% Tween® 20) were added for 1 hour at 37 °C, whereafter 50 µl TMB substrate solution was added. Subsequently, the plates were incubated at 37 °C for 30 minutes. The colour development was measured at 650 nm using an ELISA reader (Spectrafluor, TECAN) and presented as absorbance or optical density (OD).

Interpretation of the ciELISA results

The competition in the ciELISA between a free sulfonamide in the sample and the coated sulfonamide was calculated with the formula: competition (%) = (1-(A/A_o))*100 with A= absorbance of a tested sample solution and A_o the absorbance of a similar solution without sulfonamide.

A calibration curve was constructed plotting the concentration (log) of sulfachloropyridazine in the standard dilution against the competition values obtained for the corresponding dilutions. The concentration of sulfachloropyridazine in the spiked tissues was calculated by extrapolation of the competition values, obtained for these tissue samples, in the calibration curve.

Recoveries (RC, %) were calculated with the formula: (calculated concentration sulfachloropyridazine in the tissue sample / spiked concentration sulfachloropyridazine in the tissue sample) x 100. The mean tissue sample recovery, the standard deviation (SD) and standard error of the mean (SEM) were calculated. The variation coefficient of the mean recovery (CV, %) was calculated with the formula: (SD / mean)*100.

The differences between the mean specific absorbances (with pAb K3) obtained for tissue sample and buffer sample without sulfonamides were compared using the Student t-test for independent samples. The same was done for the mean background absorbance (no pAb K3).

Development of an extraction procedure

Porcine tissues spiked with sulfachloropyridazine were used to evaluate five extraction procedures differing in homogenizing method, volume of extraction buffer and treatment with kaolin (Table 9.1). During each procedure the tissue samples were diluted ten times.

Preparation of spiked tissue samples

Negative pork tissues (kidney, liver, fat and muscle) were obtained at slaughter from animals reared on an experimental unit, without sulfonamide treatment. The tissues were minced with a conventional kitchen mixer (Multi chop, CH100, 250 Watt, Braun) and stored at -80°C until use. Prior to analysis, the minced tissues were removed from the freezer. One gram of tissue was brought into a 50 ml tube (Cellstar®, Greiner bio-one, Wemmel, Belgium) and spiked with 50 µl of an appropriate sulfachloropyridazine solution. For example, to obtain

a sample spiked with sulfachloropyridazine at 100 ng/g, 50 µl of a solution of 2 µg/ml of the sulfonamide diluted in PBS was added to one gram of minced tissue.

Three tot six samples were prepared per concentration and the extracts were analysed in triplicates in the TS-ciELISA or in the PS-ciELISA. In the TS-ciELISA, sulfachloropyridazine can be detected in the range of 10 to 1000 ng/ml, and therefore spiked kidney tissues of 500, 1000 and 1500 ng/g sulfachloropyridazine were prepared, which became diluted ten times during the extraction giving extracts with 50, 100 and 150 ng/ml sulfachloropyridazine. For the analysis in the PS-ciELISA (detection range of 0.2 to 20 ng/ml), spiked samples of 200, 100 and 50 ng/g were prepared.

Choice of an appropriate extraction buffer

The solubility of different sulfonamides (sulfanilamide, sulfacetamide, sulfachloropyridazine, -pyridine, - thiazole, -diazine, -dimethoxine, -methazine, -methoxazole, -methizole and sulfisoxazole) in a carbonate-bicarbonate buffer pH 10 (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, distilled water, 1.22:1:6.62; v/v/v) was analysed. Hereto, the amount of buffer required to completely dissolve 1 mg of a given sulfonamide was determined.

Homogenization of sample and extraction buffer

Four (Table 9.1, procedure 1, 4 and 5) or 9 ml (Table 9.1, procedure 2 and 3) extraction buffer was added to one gram of spiked samples.

Two methods for homogenizing of the sample with extraction buffer were compared: a rigorous method with an ultraturax high speed mixer (Heidolph DIAX 900, Germany, speed 2, 1 min) (Table 9.1, procedure 1, 2 and 4) or a milder method by shaking with a vortex (Labinco L46, The Netherlands) at full speed for two minutes (Table 9.1, procedure 3 and 5).

Heating of the samples

Glucuronide metabolites of sulfonamides are formed in incurred tissues during storage (Haasnoot et al., 1996; Korsrud et al., 1996; Thomas et al., 1997; Alfredsson and Ohlsson, 1998; Smit et al., 1999). Since incurred samples cannot always immediately be tested, the presence of glucuronide metabolites can cause false-negative results. These metabolites were not formed in the spiked samples because the incubation time of the sulfonamides with the meat matrix was too short. Glucuronide metabolites can be reconverted into free sulfonamide

molecules by heating or under acidic condition (Thomas et al., 1997, Smit et al., 1999) or using β-glucuronidase from *Helix pomatia* (Crabbe, 2002). Therefore, heating of the samples in a warm water bath at 90°C for 15 min. (Alfredsson and Ohlsson, 1998) was included in our extraction procedures. When analysing spiked samples, no differences were noted between the analysis of heat-treated and not treated samples in the ELISA (data not shown), indicating that heat treatment had no negative effect.

Centrifugation and pH adjustment

The extracts were collected after centrifugation (10 000 g, 10 min, 4°C) of the homogenized tissue sample/buffer mixture and the pH was adjusted to pH 7-7.5 with HCl (1 M).

Kaolin treatment

The tissue samples extracted with 4 ml buffer (Table 9.1, procedure 1, 4 and 5), were further diluted by adding 500 µl PBS to 500 µl extract or were treated with kaolin. Irrespective of the procedure, the final dilution of the tissue samples was ten times.

Kaolin treatment was tested for two reasons:

1) Kaolin treatment can remove any remaining fat or other disturbing components, so decreasing the background reading in ELISA (Van den Broeck et al., 1999). The kaolin treatment was evaluated for the samples extracted with 4 ml buffer (Table 9.1, procedure 1 and 5). Hereto, 750 µl extract was mixed with 750 µl of a kaolin solution (25 % kaolin in PBS) and incubated for 30 min. at room temperature. Next, the kaolin was removed by centrifugation (10 000 g, 10 min) and 1 ml of the supernatant was diluted with 200 µl PBS.

2) When muscle samples were mixed with the extraction buffer using the ultraturax, a solid mixture was obtained. No extract could be collected after centrifugation. Mc Cracken and coworkers (2000) also noticed that the use of high speed homogenizers can give rise to sample/solvent emulsions from which no extract can be collected after centrifugation. To circumvent this problem in the present study, the samples were not centrifuged but immediately treated with kaolin after ultraturax homogenization and heating (Table 9.1, procedure 4). Hereto, 5 ml kaolin solution (25 % kaolin in PBS) was mixed with the homogenized sample (tissue/extraction buffer mixture) and incubated for 30 min. at room temperature. After centrifugation, 220 µl PBS was added to 780 µl of the supernatant.

Origin of the incurred samples

Four kidneys and 4 livers were obtained at slaughter from pigs treated with sulfachloropyridazine on an experimental unit (Department of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Ghent University). The tissues were stored at –80°C.

Table 9.1: Overview of five procedures for the extraction of sulfonamides in meat products

	Extraction procedure				
	1	2	3	4	5
volume extraction buffer	4 ml	9 ml	9 ml	4 ml	4ml
homogenization method	ultraturax	ultraturax	vortex	ultraturax	vortex
heating	yes	yes	yes	yes	yes
centrifugation	yes	yes	yes	no	yes
adjust pH	yes	yes	yes	yes	yes
kaolin	yes or no	no	no	yes	yes or no

Results and discussion

Detection of sulfonamides in the ciELISA

Specificity and limit of detection of the ELISA

The polyclonal antibodies pAb K3 were obtained by immunizing rabbits with the TS-klh conjugate. Therefore higher antibody titers were obtained in the ciELISA coated with TS-ova (TS-ciELISA) than in the ciELISA coated with PS-ova (PS-ciELISA). However, the PS-ciELISA was at least 8-times more sensitive as compared to the TS-ciELISA. In Table 9.2 the cross-reactivities (IC50 values) and limit of detection (LOD) are given for different sulfonamides (sulfadiazine, -thiazole, -chloropyridazine, -methazine, -pyridine, -dimethoxine, -merazine and sulfisoxazole) diluted in PBS, and analysed in both ciELISA. In the PS-ciELISA, all tested sulfonamides were detected at the MRL-value (100 ng/ml). The LOD for all sulfonamides, except for sulfisoxazole, was lower than 10 ng/ml. Because the ciELISA is designed to detect sulfonamides in meat samples and because samples are diluted ten times during the extraction, the detection of sulfonamides in our ciELISA should be at least ten

times more sensitive than the MRL. This requirement is fulfilled for all tested sulfonamides, except for sulfisoxazole, in the PS-ciELISA but not in the TS-ciELISA.

Table 9.2: The cross-reactivities[1] (IC50, ng/ml) and limit of detection[2] (LOD, ng/ml) for different sulfonamides analysed in the PS-ciELISA or TS-ciELISA

	PS-ciELISA		TS-ciELISA	
	LOD	IC50	LOD	IC50
Sulfathiazole	<1	3	<20	39
Sulfadiazine	2	53	<200	780
Sulfamethazine	3	326	<200	3125
Sulfadimethoxine	5	>1000	<200	4700
Sulfapyridine	<1	9	<200	400
Sulfamerazine	3	326	400	25000
Sulfisoxazole	47	>1000	400	25000
Sulfachloropyridazine	<1	9	<200	400

[1]The cross-reactivities = concentration of a sulfonamide (ng/ml) required to obtain 50% inhibition (IC50) in the ciELISA.
[2]Limit of detection = concentration read from the calibration curve at a response (absorbance) minus 3 standard deviation from the mean (n=12) for the zero standard (sample without sulfonamides)

Cross-reactivities of pAb K3 were determined for structural related molecules and drugs currently administered together with sulfonamides: diuretics (Furosemide, acetazolamide, hydrochlorothiazide, bumetanide), thiamphenicol, florphenicol, lidocaine and para-aminobenzoic acid (PS-ciELISA). The diuretics only have the $–SO_2NH-$ group in common with sulfonamides. Thiamphenicol and florphenicol have a $–SO_2-$ group, lidocaine has nothing structural in common with sulfonamides. Para-aminobenzoic acid contains the common sulfonamide *p*-aminobenzoyl ring. The polyclonals recognized *p*-aminobenzoic acid (IC50 > 10 µg/ml), but none of the other molecules. This indicates that the epitope recognized by the polyclonals contains *p*-aminobenzoyl ring. Substitution of the sulfonamide-group on the common p-aminobenzoyl ring favors the binding of the polyclonals since most sulfonamides are recognized with higher sensitivity (IC50 < 1 µg/ml; Table 9.2).

Repeatability of the ELISA

The repeatability of the PS-ciELISA (within-laboratory) was determined with the analysis of sulfachloropyridazine in buffer solution (5 different concentrations) repeated four times on the same day on the same assay (intra-assay variation), on six different assays (inter-assay variation) and on six different days (inter-day variation).

The highest variation observed was 24% for the interday variation for a concentration of 1.25 ng/ml (Table 9.3). For immunoassays, the coefficient of variation should not exceed 15 % (Crabbe, 2002). However, the level of interest is 10 ng/ml (ten times lower than the MRL). The variation around that level is acceptable. The inter-laboratory variation was not assessed.

Table 9.3: Repeatability (Coefficient of variation, %) of the PS-ciELISA with pAb K3.

	Coefficient of variation (%) for sulfachloropyridazine (ppb)				
	20	10	5	2.5	1.25
Intra-assay	2-8	2-6	1-3	2-4	4-10
Inter-assay	0-3	1-8	2-13	4-9	13-17
Inter day	8	12	12	16	24

Development of an extraction procedure

The target organs for the detection of sulfonamides in the residue control program are the kidney, liver, fat and muscle tissues (Anonymous, 1996). In practice, mostly only the kidneys and sometimes also muscles are screened for sulfonamides. Therefore, the development of an appropriate extraction procedure was first assessed on spiked kidney tissues. The extracts were analysed in the TS-ciELISA. Next, the most suitable method for the analysis of kidneys was applied on the other tissues (muscle, liver and fat) and the extracts were analysed in the more sensitive PS-ciELISA.

Choice of an appropriate extraction buffer

Most of the time, organic solvents are chosen as extraction solvent for sulfonamides, for example methanol for the extraction of sulfonamides from porcine muscle tissues (Li et al., 2000), methanol for sulfachloropyridazine in beef and lamb muscle (Spinks et al., 2001), acetonitril for sulfathiazole in porcine liver (Lee et al., 2001). For sulfamethazine, phosphate buffered saline (PBS) has often been used (Dixon-Holland and Katz, 1988; Haasnoot et al., 1996). Crooks and coworkers (Crooks et al., 2000) used a carbonate-bicarbonate buffer pH 10 for the extraction of sulfamethazine from porcine tissues. A buffer like PBS or other aqueous solutions are user-friendlier than organic solvents. Because it is the intention to develop an extraction procedure that can be applied for the extraction of all sulfonamides, a buffer in which all sulfonamides are soluble should be used. The solubility of sulfonamides is increasing with pH and temperature (Budavari et al., 1988). Therefore, the solubility of the

sulfonamides mentioned in material and methods was determined in the carbonate-bicarbonate buffer pH 10 and was at least 1 mg/ml. The MRL-value for sulfonamides is 100 ng/g (100 ng/ml). Sulfonamides present in meat at the MRL can therefore easily be extracted with the carbonate/bicarbonate buffer pH 10.

Comparison of five extraction procedures for the analysis of kidney samples

Negative kidney samples and kidney tissues spiked with sulfachloropyridazine were extracted using the five procedures, differing in homogenization method (ultraturax or vortex), in volume of extraction buffer (4 or 9 ml), and in presence or absence of kaolin treatment (Table 9.1). Recoveries were determined for each spiked sample and the mean recovery (RC % ± SEM) was calculated (Table 9.4). The reproducibility of a procedure was defined as the variation of the mean recovery (coefficient of variation, CV %).

Table 9.4: Recovery (RC % ± SEM) and reproducibility (CV %) of the extraction of sulfachloropyridazine from spiked porcine kidney tissues using five different extraction methods, and analysed in the TS-ciELISA.

Extraction procedure	Homogenization method	Kaolin treatment	RC % ± SEM	CV %
1	Turax	No	269 ± 43	49
1	Turax	Yes	98 ± 16	49
2	Turax	No	139 ± 24	31
3	Vortex	No	130 ± 8	11
4	Turax	Yes	97 ± 32	57
5	Vortex	No	95 ± 6	10
5	Vortex	Yes	67 ± 7	18

Lower recoveries were obtained when kaolin treatment was included in the extraction procedure (procedure 1 and 5). Samples prepared following procedure 1 with kaolin treatment resulted in a mean recovery of 98 % as compared to 269 % without kaolin. For procedure 5, an RC of 67 % was found after kaolin treatment as compared to 95 % for the extraction carried out without kaolin (Table 9.4).

Using a vortex resulted in a lower variation in the mean recovery (CV % between 10 and 18 %, procedure 3 and 5) and thus provided a higher reproducibility than the procedures using the ultraturax mixer (CV % between 31 and 57 %, procedure 1, 2 and 4) (Table 9.4). The large variation with the ultraturax mixer, is most likely due to direct adherence of sample

to the mixer resulting in a variable loss when the mixer is removed. Furthermore, this contact increases the risk for cross-contamination (McCracken et al., 2000). No loss of sample nor cross-contamination occurs during the homogenization with the vortex mixer. Loss of sample causes a concentration effect: the analyte is distributed in a smaller volume, increasing the concentration and thus leading to recoveries exceeding 100 %.

In a previous study in which an extraction procedure for the detection of penicillins in meat samples was developed, kaolin treatment was found to be very efficient to diminish background signals in ELISA (unpublished data). It was assessed if such a treatment was required for the analysis of samples in the sulfonamide-specific TS-ciELISA. Hereto negative kidney tissues were extracted with the five procedure (Table 9.1) and the extracts were analysed in the TS-ciELISA. Background absorbances (without pAb K3) and specific absorbances (with pAb K3) obtained for the extracts were compared to the background and specific absorbances respectively obtained for PBS. It was found that kaolin treatment did not influence the absorbances. For all procedures, irrespective the application of kaolin, no significant difference ($p > 0.05$) was noted between the specific absorbance in extract compared to the response in PBS. Furthermore, higher background signals were measured when the ultraturax mixer was used (background absorbances > 0.1000, procedure 1 and 4) compared to vortex mixing (background absorbances < 0.0800, procedure 5).

As conclusion, procedure 3 and procedure 5 without kaolin treatment were found to be the most suitable procedures for the extraction of sulfachloropyridazine from kidneys when analysing the extracts in the TS-ciELISA, because vortex homogenization provided a higher reproducibility and lower background signals than the procedures using the ultraturax mixer.

Comparison of both extraction procedures with vortex homogenization for the analysis of kidney, fat, liver and muscle tissues

In a second step procedure 3 and 5 were assessed on more tissues. Therefore, extracts of porcine muscle, liver, kidney and fat tissues, spiked with sulfachloropyridazine, were prepared with both procedures. Procedure 5 was applied with and without kaolin treatment, except for the kidney samples, which were only extracted without kaolin treatment. The extracts were analysed in the PS-ciELISA.

The recoveries obtained for procedure 5 varied considerably between the different tissues (Table 9.5). Recoveries between 59 % and 184 % were noticed without kaolin treatment, and between 59 % and 374 % with kaolin treatment. Furthermore, procedure 5

showed a low reproducibility for fat and muscle samples (CV between 37 % and 61 %), whereas using procedure 3, the recoveries were comparable for the different tissues (107 % for kidney, 109 % for liver, 119 % for fat and 116 %) for muscle tissues, with a higher reproducibility for fat (CV = 16 %) and muscle (CV = 23 %) (Table 9.5).

Table 9.5: Recovery (RC % ± SEM) and reproducibility (CV %) of the extraction of sulfachloropyridazine from spiked porcine tissues (muscle, fat, liver and kidney) using extraction procedure 3 or 5 (with and without kaolin treatment) and analysed in the PS-ciELISA.

	Procedure 3	Procedure 5 without kaolin	Procedure 5 with kaolin
	RC % ± SEM (CV %)		
Kidney	107 ± 14 (22)	98 ± 10 (18)	-[1]
Liver	109 ± 5 (8)	59 ± 2.5 (14.5)	59 ± 2 (13)
Fat	119 ± 4.5 (16)	140 ± 20 (61)	374 ± 33 (38)
Muscle	116 ± 6 (23)	184 ± 20 (45)	116 ± 10 (37)

[1]For the kidney tissues, no kaolin was applied for procedure 5.

In contrast to the TS-ciELISA, a significant matrix effect was observed in the PS-ciELISA for most tissues as evidenced by significant differences between the specific absorbances for extracts in comparison with PBS. This was not the case for fat extracts prepared using procedure 5 without kaolin (not significant, $p = 0.36$) and muscle extracts prepared with procedure 5 with kaolin treatment (not significant, $p = 0.08$). This highlights the importance of an appropriate matrix control (negative tissue extract) for the calculation of competition values of spiked and incurred samples analysed in the ciELISA.

As for the TS-ciELISA, kaolin treatment did not improve the efficiency of sample analysis in the PS-ciELISA. No background differences between tissue extracts and PBS were noted for kidney extracts prepared with procedure 3 and 5 ($p = 0.45$ and $p = 0.08$, respectively), and for liver prepared with procedure 3 and 5 with kaolin treatment ($p = 0.71$ and $p = 0.10$ respectively). In contrast, significantly different background signals were seen between tissue extract and PBS for fat and muscle extracts prepared with both procedures. However, even when significantly different, the background absorbances never exceeded 0.0900 for most extracts nor for PBS, with the exception of procedure 5 without kaolin applied on fat tissue (absorbance = 0.1222), and procedure 3 for liver (0.0962), and therefore it was concluded that kaolin treatment will not improve the efficiency of the sample analysis in the PS-ciELISA.

Thus, when analysing samples in the PS-ciELISA, procedure 3 was preferred for the extraction of sulfachloropyridazine from porcine kidney, liver, fat and muscle tissues.

Care must be taken when comparing recoveries obtained in different studies. The recovery is not only dependent on the homogenization method, but also on the extraction buffer, the extracted analyte, the concentration of analyte and de detection method.

Thomas and coworkers (1997) reported different recoveries for sulfathiazole, sulfachloropyridazine, sulfamethazine and sulfaquinoxaline extracted with the same procedure from spiked porcine liver tissues and analysed in LC-MS/MS (recovery = 27%, 69%, 84% and 33%, respectively). These differences were subscribed to the fact that the efficiency of extracting different sulfonamides is pH dependent. So a procedure using a single extraction pH cannot quantitatively recover the full range of sulfonamides with the same efficiency. At the moment, there are no data available on the recovery of other sulfonamides using procedure 3.

Spinks and coworkers (2001) analysed porcine liver, muscle and kidney tissues spiked with sulfachloropyridazine, and extracted with methanol and/or PBS, in ELISA. Unfortunately, they did not mention the recovery data.

In the present study, the same extraction buffer was used as Crooks and coworkers (1996). They extracted sulfamethazine from homogeneous lyophilised pig tissues for the detection in HPLC. Recoveries of at least 80% were mentioned. However, the use of the extraction buffer is only one step in the sample preparation for the HPLC analysis and therefore, comparison with present results is not relevant. There are no data cited in literature about the recovery of sulfachloropyridazine from porcine tissues using a carbonate/bicarbonate extraction buffer.

Decision limit and detection capability

In conclusion of preceding paragraph, procedure 3 can be applied for the extraction of sulfachloropyridazine in kidney, muscle, liver and fat tissues. In order to use the ELISA for screening purposes, the decision limit CCα and detection capability CCβ were determined according to the Commission Decision 2002/657/EC (Anonymous, 2002). Twenty negative porcine kidneys were fortified with sulfachloropyridazine at the MRL (100 ppb), extracted following procedure 3 and analysed in the PS-ciELISA. CCα and CCβ were calculated using the average competition (X_{MRL}) and standard deviation (SD) obtained for the spiked samples

($CC\alpha = X_{MRL} + 1.64*SD$; $CC\beta = CC\alpha + 1.64*SD$). According to the Commission Decision 2002/657/EC, at least 20 samples spiked at the CCα should be analysed to determine CCβ. However, CCβ was determined using the calculated CCα and the SD of X_{MRL}, in the supposition that approximately the same SD for the average competition for 20 spiked samples at CCα would be obtained as for spiked samples at the MRL. The resulting values are therefore an approximately estimation for CCα and CCβ. The corresponding concentrations were obtained by plotting the competition values in a calibration curve of sulfachloropyridazine: CCα = 94 ppb, CCβ = 129 ppb.

During screening, a sample should thus be considered non-compliant when the concentration is higher than the CCβ, compliant when lower than CCα and suspected when between CCα and CCβ.

Analysis of incurred samples

The extraction procedure for the analysis of porcine tissues in the PS-ciELISA was developed using spiked samples. The decision limit and detection capability for porcine kidneys were also determined using spiked samples containing penicillin at the MRL, according to Commission Decision 2002/657/EC (Anonymous, 2002). However, attention must be taken when validating a method based on fortified samples. An extraction protocol optimized using fortified samples will not necessarily be efficient for incurred samples. The efficiency of the extraction procedure to extract analytes from the tissue matrix cannot be determined using fortified samples because the interaction between analyte and the sample matrix is different compared to incurred samples (McCracken et al., 2000). Therefore, 4 kidneys and 4 livers from pork experimentally treated with sulfachloropyridazine were analysed in the PS-ciELISA and compared with the analysis of the same samples using LC-MS/MS method. The LC-MS/MS analyses were performed at the Department of Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Ghent University (De Baere et al., 2000).

The concentrations obtained with the ELISA and the LC-MS/MS method are shown in Table 9.6, the correlation between both methods is established in Table 9.7. The concentration sulfachloropyridazine in the 4 liver samples was higher in ELISA as compared to the LC-MS/MS method. However, the correlation between both methods for the liver analyses was satisfactory ($r^2 > 0.85$; Table 9.6). Supposing that the LC-MS/MS method gave the correct concentration, this means that the ELISA will produce false non-compliant results rather than

false-compliant results. The concentrations of the kidneys prepared following extraction procedure 5 are much lower as compared to the concentrations obtained with procedure 3 and with LC-MS/MS. The correlation between the ELISA and the LC-MS/MS for the kidney analyses is higher when using extraction procedure 3 as compared to procedure 5 (r^2 = 0.77 en 0.52, respectively; Table 9.7).

A possible explanation for the higher concentrations obtained using the ELISA as compared to the LC-MS/MS method, could be the use of a different extraction buffer. For the ELISA, an aqueous buffer (carbonate/bicarbonate pH 10) is used while organic solvents (acetone and ethylacetate) for the LC-MS/MS analysis. Muldoon and coworkers (2000b) compared HPLC with ELISA for the determination of sulfadimethoxine concentration in chicken livers. When an organic solvent was used for sample preparation before ELISA analysis, high correlations were found between both methods (r^2= 0.97). When an aqueous extraction method was used, higher concentrations were detected in ELISA as compared to HPLC. Moreover, the ELISA results did not correlate well with the HPLC results (r^2= 0.61). They deduced that the ELISA detected other cross-reactive compounds such as sulfadimethoxine-protein conjugates and free metabolites of the sulfonamide present in the aqueous extract. They concluded that the amount of sulfadimethoxine detected in incurred samples was not only dependent of the detection method but also of the extraction procedure. The importance of the extraction procedure in sample analysis is also demonstrated in our results. For most of the samples, in particular livers, higher amounts of sulfachloropyridazine were detected after extraction using procedure 3 as compared to procedure 5. The recovery using procedure 5 is also lower than the recovery obtained with procedure 3 (Table 9.5), indicating that there is a loss of analyte during the extraction.

Table 9.6. Concentration sulfachloropyridazine in liver and kidney tissues (in ppb) analysed with the LC-MS/MS method and in the PS-ciELISA.

	Sample	LC-MS/MS	ELISA		
			procedure 3	procedure 5 (without kaolin)	procedure 5 (with kaolin)
Liver	1	4.7	28.2	34.0	8.3
	2	50.0	260.5	111.6	64.6
	3	84.0	231.1	126.0	120.1
	4	150.0	726.7	416.6	641.8
Kidney	1	24.0	45.0	4.3	
	2	71.0	250.7	22.9	
	3	773.0	734.5	50.0	
	4	5232.0	1140.7	55.9	

Table 9.7. De correlation (r^2) between LC-MS/MS and the PS-ciELISA using pAb K3 for the analysis of porcine kidney and liver

	procedure 3	procedure 5 (without kaolin)	procedure 5 (with kaolin)
Liver	0.88	0.89	0.85
Kidney	0.77	0.52	

In conclusion, based on the comparison of different extraction procedures for analysis of spiked porcine tissue samples in the PS-ciELISA, based on the correlation between the ELISA and LC-MS/MS for the analysis of incurred samples, and because it is more suitable to have one extraction procedure for different tissues, extraction procedure 3 is preferred for the analysis of porcine kidney and liver tissues in the PS-ciELISA. In further work, the detection of other sulfonamides using this sample preparation in combination with the analysis of the extract in the PS-ciELISA should be assessed.

Acknowledgements: This study is supported by the Ministry of Public Health, Brussels, Belgium and by the Institute for Veterinary Inspection (IVK). The technical assistance of Els De Vogelaere was greatly appreciated. We thank Roeland Mijngheer for the synthesis of the sulfonamide derivatives.

Part V

General discussion and conclusion

Chapter 10: General discussion and conclusion

This study started 7 years ago, when the demand for sensitive methods for the detection of all kinds of antimicrobials in food products became more and more important. At that time, microbiological inhibition assays were mostly applied for the screening of food products of animal origin. These tests have a broad-spectrum specificity and are therefore useful for screening purposes. Suspected non-compliant samples are further screened using other microbiological assays or using immunoassays to identify the inhibitory substance or the group to which it belongs. Finally, the analyte is identified and quantified using a confirmatory method. The aim of this thesis was to investigate the possibility of developing a group-specific screening test using antibodies. The idea was to develop antibodies specific for the common structure of a group of antimicrobials, for example the group of penicillins or the group of sulfonamides. Such antibodies should allow the development of an immunochemical assay, like the enzyme-linked immunosorbent assay (ELISA), which then, in combination with an appropriate extraction procedure, could be applied for the detection of penicillins, or sulfonamides in meat samples.

10.1. Development of group-specific antibodies

10.1.1. Immunogens and immunization

An important part of this thesis deals with the development of monoclonal and polyclonal antibodies specific for the group of penicillins and monoclonals and polyclonals specific for the group of the sulfonamides. The aim was to find a suitable strategy for the induction of such antibodies.

First, good immunogens had to be constructed because penicillins and sulfonamides are too small to elicit an immune response. Hereto a penicillin and a sulfonamide, respectively, were conjugated to a carrier protein. The conjugations were performed in such a way that the common structure of penicillins and sulfonamides remained free for binding antibodies (Chapter 4 and 8; Cliquet et al., 2001; Cliquet et al., 2003a).

10.1.1.1. Penicillins

Ampicillin was chosen as the penicillin to be coupled to different carrier-proteins (bovine serum albumin, chicken ovalbumin and thyroglobulin), because of the free amino

group in its side chain. Different coupling methods were compared: two methods using a cross-linker (glutaraldehyde or a succinimide ester), one carbodiimide-mediated coupling method and one method without any cross-linker or mediator molecule (physiological binding) (Chapter 4).

The hapten-carrier conjugates were characterized by determining the number of penicillin molecules per carrier molecule and by their capacity to induce polyclonal antibody responses in mice (Chapter 4). For the penicillin-carbodiimide mediated conjugates, no appropriate method was available for determining the number of ampicillin molecules per carrier molecule. For the other conjugates, the number of hapten molecules coupled to one protein molecule could be measured. These numbers were similar to or slightly higher than those obtained by other investigators *(*Van Regenmortel, 1988; Katsutani and Shionoya, 1993; Märtlbauer, 1993*).*

The antibody response against non-physiological conjugates was moderate to low and no penicillin-specific monoclonals were obtained (Chapter 4, Cliquet et al., 2001). Usleber and coworkers (2000) immunized rabbits and mice with ampicillin or benzylpenicillin coupled to albumins using glutaraldehyde, mixed anhydride or an active ester as cross-linker and also found the conjugates being non- or weakly immunogenic. Only three out of nine rabbits and none of the twelve mice immunized with the glutaraldehyde conjugate produced specific antibodies. Similar weak responses were observed coupling ampicillin to keyhole limpet hemocyanin (klh) with glutaraldehyde. This conjugate was non-immunogenic in rabbits and resulted in an immune response in only two of twelve mice. These data demonstrate the importance of immunizing enough animals before making conclusions. In addition, results about the conjugate indicate that despite using genetically selected strains (Balb C mice) there is still diversity in the immune response between different animals. In our work, we assessed the immunogenicity of penicillin conjugates using three to four animals.

As expected from literature, a high antibody response was obtained against the physiological ampicillin-protein conjugates, (Chapter 4; Dietrich et al, 1998; Cliquet et al., 2001). Such conjugates are also formed *in vivo* after intake of penicillin and are responsible for the penicillin hypersensitivity reactions (Katsutani and Shionoya, 1993). Fusion experiments with the splenocytes of one mouse resulted in four penicillin-specific monoclonals. Two of them, mAb 19C9 and mAb 9H3, were selected for further investigation.

10.1.1.2 Sulfonamides

In our first approach for the induction of sulfonamide-specific antibodies, sulfanilamide was chosen for conjugation because it is the common structure of all sulfonamides and it does not have a "disturbing" side group. Sulfanilamide was linked to proteins using glutaraldehyde or a succinimide ester as cross-linker or by diazotation. As for the penicillins, the sulfonamide-carrier conjugates were characterized by determining, if possible, the number of sulfonamide molecules per carrier molecule and by their capacity to induce polyclonal antibody responses in mice. While the non-physiological penicillin conjugates induced moderate to low antibody responses, no immune response at all was obtained using the sulfanilamide-protein conjugates cross-linked with glutaraldehyde or a succinimide ester. On the other hand, high antibody titers were obtained after immunization with the diazotation conjugates. But these antibodies were highly specific for the bound sulfanilamide molecule and did not recognize free sulfanilamide or other sulfonamides. Other investigators have also reported the use of the diazotation reaction to construct sulfonamide-protein conjugates for the induction of antibodies against one particular sulfonamide (sulfamethazine, sulfamerazine and sulfathiazole). They also obtained antibodies highly specific for the bound sulfonamide molecule, but their antibodies were always able to recognize the free molecule (Fleeker and Lovett, 1985; Garden and Sporns, 1994; Sheth et al., 1990). Sulfanilamide does not have a side chain like sulfamethazine, -merazine and –thiazole and this could be the reason why we did not obtain sulfanilamide-specific antibodies. The epitope in the sulfanilamide conjugates is probably too close to the linkage side and thus sterically inhibited by the carrier protein for the binding of antibodies.

Our second approach was the use of sulfonamide-protein conjugates in which the sulfonamide is linked at its side chain, leaving the common group unchanged and thus available for the immune system. Sulfonamide derivates with a large side chain containing a carboxyl group were coupled to carrier proteins by a carbodiimide-mediated reaction. Immunizations with the N-sulfanilyl-4-aminobenzoic acid-protein conjugates (S-conjugates) led to high antibody production, but all of them were highly specific for the conjugates. The use of a sulfathiazole derivative (N1-[4-(carboxymethyl)-2-thiazolyl]-sulfonamide) (TS) (Sheth and Sporns, 1991) and another sulfonamide derivative with a larger side chain (N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide) (PS) (Assil et al., 1992) was more successful. Haasnoot et al. (2000a; 2000b) used the same approach and found that immunization of mice with PS-klh led to antibodies that recognized sulfonamides

(sulfamethazine, -merazine, -diazine, -dimethoxine) that were not well recognized after TS-klh immunization. Therefore, we tried to direct the immune response towards recognition of a broader range of sulfonamides by immunizing the animals alternately with TS- and PS-protein conjugates. As expected from the results of Haasnoot et al. (2000a; 2000b), immunization with PS-bsa or PS-klh did not induce a high antibody titer. On the other hand, the alternate immunization with TS- and PS-conjugates induced antisera with a broader specificity than immunization with TS-conjugates only. The mice immunized with TS-klh only, had lower antibody binding to sulfonamides containing a pyrimidinyl group (sulfamethazine, -merazine, -diazine) or a pyridinyl group (sulfapyridine) as compared to the binding of antibodies derived from the alternately immunized mice (Chapter 8, Cliquet et al., 2003a).

10.1.2. Production of monoclonal and polyclonal antibodies

10.1.2.1. Penicillins

For the production of the monoclonals, an intravenous final boost gave antibodies with a higher specificity and affinity than an intraperitoneal one (Chapter 4; Cliquet et al., 2001). Indeed, the intravenous injection will result in a rapid and strong response of splenic lymphocytes that subsequently will be used for fusion, as the antigen will be captured quickly in the spleen (Harlow and Lane, 1988). But, in our experience, intravenous injection can sometimes fail, probably because it is a delicate manipulation. It is difficult to insert a needle in a tail vein. To overcome this risk, a final boost consisting of an intravenous and an intraperitoneal injection was performed 4 days before fusions instead of three (for intravenous injections) or five (for intraperitoneal injections) days. This procedure was applied to all fusion experiments that were done to obtain sulfonamide-specific monoclonal antibodies. In our hands, this procedure works excellent.

After fusion experiments with splenocytes of a mouse immunized with the physiological ampicillin-protein conjugates, two mAbs were selected. These monoclonals (mAb 19C9 and 9H3) bind to ampicillin (100%), amoxicillin (964%, 212% respectively), benzylpenicillin (43%, 181% respectively), oxacillin (24%, 19 % respectively), dicloxacillin (14%, 21% respectively) and cloxacillin (9 %, 17 % respectively) (data obtained in an antigen ciELISA). Hydrolysed penicillins (open β-lactam ring) are less well recognized by both monoclonals, whereas the structural related cephalosporins (dihydrothiazin instead of thiazolidin ring) are not recognized at all. Therefore, it is concluded that the monoclonals are

specific for the thiazolidin ring of the penicillin molecule but that both the β-lactam ring and the thiazolidin ring are important for the detection of penicillins by the monoclonals (Chapter 6; Cliquet et al., 2004).

In literature only two other monoclonal antibodies (mAb 1D1 and mAb 3B5; Usleber et al., 2000) are mentioned as being penicillin-specific. These monoclonals show a high affinity for ampicillin (100 %) and amoxicillin (187 %, 108 % respectively) and a lower for benzylpenicillin (31%, 8.6 % resp.), oxacillin (14 %, 4.5 % respectively), cloxacillin (30 %, 4.8 % respectively) and dicloxacillin (44 %, 1.7 % respectively) (data obtained in an antigen ciELISA). No cross-reactivities with hydrolysed penicillins or cephalosporins were observed. These cross-reactivity profiles indicate that the monoclonals are specific for the intact β-lactam ring and that the amino-group in the side chain is important (Dietrich et al., 1998; Usleber et al., 2000). Our mAbs recognize the thiazolidin ring of the penicillin structure. However, it is assumed that the β-lactam ring is also important for the binding of these monoclonals since hydrolysed penicillins are recognized with lower affinity. Usleber and coworkers (2000) obtained their monoclonals after immunization with a weak immunogenic glutaraldehyde coupled ampicillin-klh conjugate whereas we used the highly immunogenic physiological penicillin-protein conjugates. It can be questioned if such antibodies could have been obtained with our immunogen. In the present thesis, hybridomas were selected based on the screening of their supernatant in an antigen ciELISA. Only the hybridomas generating a high absorbance as well as a high inhibition in the presence of non-hydrolysed ampicillin were selected. Doing this, no selection is made between antibodies specific for the open ring and the antibodies specific for the intact β-lactam ring. To differentiate between these two groups of antibodies, inhibition of the antibodies in the presence of hydrolysed ampicillin should also be tested. Antibodies expressing inhibition in presence of the native ampicilline but not in presence of the hydrolysed molecule are consequently specific for the intact β-lactam ring. Similarly, antibodies expressing inhibition in the presence of hydrolysed penicillins but not in the presence of the native penicillin are consequently specific for the open β-lactam structure. In the present study, we have selected antibodies specific for the thiazolidin ring recognizing both intact and hydrolysed penicillins. Moreover, using an isoxazolyl penicillin instead of ampicillin would probably allow to select hydridomas producing antibodies with higher affinity for these penicillins as compared to hybridomas selected using ampicillin. These results demonstrate the importance of well-defined screening strategies.

The limit of detection (LOD) of amoxicillin, ampicillin, benzylpenicillin and cloxacilline in buffer solution analyzed in an antigen ciELISA using mAb 1D1 ranged from 12 to 130 ng/ml (Dietrich et al., 1998). This ELISA is much more sensitive than our ELISA using mAb 19C9 or mAb 9H3 in which the LOD of almost all tested penicillins is higher than 200 ng/ml, except for amoxicillin using mAb 19C9 (LOD = 18 ng/ml) (Chapter 6, Cliquet et al., 2004).

Based on the results obtained for mice, three rabbits were immunized with the physiological penicillin-albumin conjugates in order to obtain polyclonal antibodies. In contrast to the mAbs, the pAbs were more specific for the hydrolysed penicillins than for the native penicillin (Chapter 5; Cliquet et al., submitted). Cross-reactivities of the rabbit pAb with the structural related cephalosporins were only noticed for cefaclor when analysed at high concentration (IC50 cefaclor approx. 5000 ng/ml as compared to IC50 < 200 ng/ml for all tested penicillins). It is not clear why these pAbs recognize cefaclor but not cephalexin or cephadrin. These three cephalosporins mainly differ in the substitution on the dihydrothiazin ring (-Cl for cefaclor *vs* $-CH_3$ for cephalexin and cephadrin) (Budavari, 1988). Probably the polyclonals mainly recognize the upper part of the thiazolidin and dihydrothiazin ring, since they do not recognize clavulanic acid (oxygen instead of sulphur). Other cephalosporins have larger side groups causing sterical hinder to the antibodies for binding to the upper part of the dihydrothiazin ring.

The detection of penicillins in ELISA was more sensitive using the pAb than the mAb. All tested penicillins could be detected at their MRL in the antibody ciELISA using pAb K2 (Chapter 5; Cliquet et al., submitted). The MRL concentration was not reached for any penicillin when mAb 9H3 was used (LOD > 50 ng/ml), however with mAb 19C9, only amoxicillin (LOD = 18 ng/ml) could be detected at the MRL.

Usleber and coworkers (1998) obtained a penicillin-specific rabbit antiserum after immunization with a glutaraldehyde coupled ampicillin-albumin conjugate. The ELISA developed with these antibodies was not sensitive enough and detected the intact, non-hydrolysed penicillins. The MRL is set up for this structure. With our antisera, a sensitive method is developed that mainly detects the degradation products, which are not taken into account in the MRL. However, the detection of the hydrolysed penicillin can be important. Although no scientific evidence is provided that people can have adverse reactions after intake of penicillin through food products, the risk in case of already sensitised individuals may not be underestimated (Dewdney, 1991). Ormerod et al. (1987) reported the induction of adverse reaction after intake of milk containing benzylpenicillin (6 ppb) by individuals who

were already hypersensitive for penicillins. De Baere and coworkers (2002) detected high amounts of metabolites after administration of amoxycillin to pigs, in contrast to what is usually assumed. Furthermore, spontaneous hydrolysis of penicillins will occur during sample handling and will always give an underestimation of the penicillin concentration in the original sample. The MRL for penicillins was one of the first established levels (Anonymous, 1990). At that time, no attention was paid to the metabolites of the molecule. Phillips and coworkers (2004) remarked that penicillin would nowadays probably not be approved for use in human medicine given its neurotoxicity, the high incidence of allergy and the common occurrence of resistance. It is about time to review this MRL.

10.1.2.2 Sulfonamides

The mAbs obtained after fusion experiments with the splenocytes of mice immunized with TS- and/or PS-protein conjugates could be divided, based on their cross-reactivities, into three groups, independently from which mouse they were deduced (Chapter 8; Cliquet et al., 2003a). This means that the alternate immunization with two different immunogens with the intention to broaden the specificity of the antibodies, indeed improved the broad-specificity of a polyclonal serum, but not of individual mAbs. Consequently, to obtain broad-specific mAb, it would be sufficient to immunize with TS-klh, and to screen the hybridomas in an ELISA coated with PS-ova.

To induce polyclonal antisera, rabbits were immunized with the most successful sulfonamide immunogen. As for penicillins, the detection of sulfonamides was more sensitive using the pAb-based ELISA as compared to the mAb-based ELISA.

Cross-reactivities of mAb 3B5B10E3 and pAb K3 for structural related molecules or drugs that are administered together with sulfonamides to animals were determined (PS-ciELISA) (Chapter 8 and 9). The monoclonal recognized four diuretics (furosemide, acetazolamide, hydrochlorothiazide, bumetanide; IC50 >10 µg/ml), but none of the other tested molecules (thiamphenicol, florphenicol, lidocaine and *p*-aminobenzoic acid). The diuretics only have the $–SO_2NH-$ group in common with sulfonamides. Thiamphenicol and florphenicol have a $–SO_2-$ group, *p*-aminobenzoic acid contains the common sulfonamide *p*-aminobenzoyl ring and lidocaine has nothing structural in common with sulfonamides. This indicates that the epitope recognized by the monoclonal included the $–SO_2NH-$ group. Substitution of this sulfonamide-group on a benzene ring favors the binding of the monoclonal since sulfonamides are recognized with higher sensitivity (IC50 <1 µg/ml). On the other hand, the polyclonals recognized *p*-aminobenzoic acid (IC50 >10 µg/ml), but none

of the other molecules. This indicates that the epitope recognized by the polyclonals contains the *p*-aminobenzoyl ring. Substitution of the sulfonamide-group (-SO_2NH-) on the common *p*-aminobenzoyl ring favors the binding of the polyclonals since most sulfonamides are recognized with higher sensitivity as compared to *p*-aminobenzoic acid (IC50 sulfonamides <1 µg/ml).

Several studies have been published on the development of group-specific sulfonamide antibodies. Some studies followed the same strategy as we did (Sheth and Sporns, 1991; Assil et al., 1992; Muldoon et al., 1999; Haasnoot et al, 2000a; Haasnoot et al., 2000b). The most sensitive monoclonal, mAb 27G3, was obtained by Haasnoot and coworkers (2000a; 2000b) and showed 50 % inhibition (IC50) with 18 tested sulfonamides at values less than 10 µg/ml, and with eight at concentrations below 0.1 µg/ml. Nevertheless, the most relevant sulfonamides sulfamethazine, sulfatroxazole and sulfachloropyrazine were not detected at the MRL value (100 ppb or 0.1 µg/ml). The improvement of the cross-reactivity profile of that monoclonal by genetic engineering has also been published. Korpimäki and coworkers (2002, 2003) obtained a mutant M3.4. with higher affinity for the tested sulfonamides than the wild-type mAb 27G3.

Li and coworkers (2000) used synthetic sulfonamides not described by anyone else to produce rabbit polyclonals. They reported IC50 values in the range of 0.2 to 2.2 µg/ml for sulfamethazine, sulfadimethoxine, sulfathiazole, sulfadiazine and sulfaquinoxaline in buffer solution analyzed in a competitive ELISA using the pAb. Spinks et al. (1999) carried out molecular modeling studies on the sulfonamide structure and noticed that the molecule has a characteristic bend around the tetrahedral –SO_2- group. They deduced that the recognition of the common sulfonamide structure would be maximal for these drugs where the bend had the greatest angle. Consequently, cross-reactive antibodies could possibly be obtained using a sulfonamide as hapten with a more planar structure (sulfacetamide) or a larger bend (sulfachloropyridazine). Despite this interesting hypothesis, immunization with such conjugates did not lead to antiserum with a broad-specificity for sulfonamides.

Beside the mutant M3.4, two other interesting monoclonals were reported in literature: the sulfonamide-specific rat mAb 21C7 obtained by coincidence during the development of sulfamethazine-specific monoclonals (Kohen et al., 2000) and the monoclonal used in the Biacore kit for sulfonamides (Qflex kit) (Mc Grath et al, 2004). The development of the latter one has not been published. Bienenmann-Ploum and coworkers (2004) have compared a biosensor assay using the mutant M3.4. and the mAb 21C7 with the Qflex kit for the detection of 26 different sulfonamides and metabolites in chicken serum. The mutant M3.4 was found

to be the most sensitive toward most of the sulfonamides, whereas the Qflex kit detected 5 sulfonamides registered for application in poultry (sulfamethazine, sulfamethoxazole, sulfaquinoxaline, sulfachloropyridazine, sulfadiazine) within the narrowest measurement range (LOD between 19 and 62 ng/ml for the Qflex kit versus LOD between 4 and 82 ng/ml for mutant M3.4.). For group-specific screening assays it is more interesting to detect the analytes in a narrow range because the decision of compliance is made by comparing to a reference analyte. A small range will avoid too much false compliant and false non-compliant results.

Our monoclonal 3B5B10E3 detects most of the tested sulfonamides (sulfathiazole, sulfamethoxazole, sulfachloropyridazine, sulfadiazine) in buffer solution in a range of less than 1 to 80 ng/ml, and thus below the MRL (100 ng/ml). However, sulfamethazine and sulfamerazine cannot be detected at the MRL with this monoclonal (Chapter 8; Cliquet et al, 2003a). This problem can be solved using polyclonal antisera or a combination of monoclonal antibodies, each with different specificities. Haasnoot et al (2000c) could detect sixteen sulfonamides in the BIAcore 2000 biosensor using a combination of three group-specific monoclonal antibodies.

10.1.3. Conclusions

The main problem for the induction of penicillin-specific antibodies was the development of a suitable immunogen. Positive results were obtained with the physiological conjugates. In these conjugates, the β-lactam ring of penicillins is opened; as a result, an immune response is induced against an epitope different from the closed β-lactam structure of penicillins and cephalosporins. A screening of hybridomas to select antibodies recognizing the intact β-lactam ring has not been performed. Consequently, penicillin-specific, but not β-lactam specific antibodies were selected.

The main problem for the induction of sulfonamide-specific antibodies was the large structural difference between the different molecules due to variations in the side chains. A monoclonal able to recognize the sulfonamide core in all the different sulfonamide molecules is therefore difficult to find.

For both the penicillins and the sulfonamides, the detection of the analytes using the polyclonal antibodies was more sensitive than using the monoclonals. Booman (1988) already discussed that the affinity of monoclonals is usually far below the avidity of the

corresponding polyclonal antisera, since polyclonals contain different antibodies against all the haptens. The sensitivity of detection using these polyclonals is related to their avidity.

It can be questioned if the production of a group-specific antibody is more a trial and error process in combination with the coincidence of the induction of that rare, specific antibody. Strong immunogens, like the physiological penicillin-protein conjugates or the sulfonamide-protein conjugates (azocasein, TS- and S-conjugates), resulted in high titers of penicillin-specific or sulfonamide-specific antisera in all immunized animals, but fusion experiments have not necessarily provided group-specific antibodies. On the other hand, weak immunogens induced generally low titers. But in some animals moderate to high titers can be found and fusion experiments can result in some rare group-specific antibodies. For instance the ampicillin-klh glutaraldehyde conjugate used by Usleber and coworkers (2000) only induced an immune response in two of the twelve immunized mice. Fusion experiments using the splenocytes of these two responding mice resulted in two rare penicillin-specific and sensitive monoclonal antibodies. The same is true for the study of Muldoon et al. (1999). They immunized 5 mice with a sulfonamide derivative coupled to klh. Only one mouse produced high titers of antibodies able to recognize other sulfonamides. The fusion experiment resulted in only two wells of the thirty 96-wells microtiter plates containing hybridoma colonies that recognized more than one sulfonamide and resulted in one monoclonal antibody specific for the group of sulfonamides. Thus, it can be worthwhile to do more research on such weak immunogens and their induction of group-specific antibodies, instead of looking for strong immune responses. Another interesting study was described by Kohen and coworkers (2000). They obtained by chance a sulfonamide cross-reactive monoclonal antibody using as immunogen sulfamethazine coupled by diazotation to BSA. Such immunogens would normally induce sulfamethazine-specific antibodies, as observed by other investigators (Fleeker and Lovett, 1985; Sheth et al., 1990; Garden and Sporns, 1994). This mAb was used to develop a biosensor assay (Haasnoot et al, 2003). Eight sulfonamides (sulfamethazine, -diazine, -merazine, -chloropyazine, -sulfisoxazole, -sulfachloropyridazine, -sulfatroxazole, -sulfathiazole) were detected in buffer solution and in diluted chicken serum at concentrations between 7 to 20 ng/ml.

Thus, the choice of an appropriate immunogen is critical for the development of group-specific antibodies. However, it must be taken into account that the potential of inducing a high immune response is not necessarily the most important selection parameter.

10.2. Influence of the test system on the sensitivity of detection

The cross-reactivities and sensitivities of antibodies not only depend on the way they were induced or selected, but also on the test system they are used in. During our research, two immunochemical methods were applied: a competitive inhibition ELISA (ciELISA) and the optical biosensor of BIAcore (biosensor).

The **penicillin-specific monoclonals** mAb 19C9 and mAb 9H3 were analysed using a ciELISA coated with antigen (antigen ciELISA) and using the biosensor (Chapter 6; Cliquet et al, 2004). Both monoclonals recognize ampicillin, amoxicillin and benzylpenicillin better than oxacillin, cloxacillin and dicloxacillin. The latter ones have a larger side chain, possibly causing sterical inhibition for the binding of the monoclonals to the common penicillin nucleus. No ciELISA coated with antibodies (antibody ciELISA) could be developed using the monoclonal antibodies, probably because of changes in their conformation due to the immobilization on the plate. With both antibodies, but especially with mAb 9H3, oxacillin, cloxacillin and dicloxacillin were better recognized in the biosensor system than in the ELISA. In ELISA, it was not possible to detect these penicillins at concentrations below 1 µg/ml. A possible explanation is that in ELISA the binding of the antibodies to the coated molecule is favoured above its binding to the antigens in solution for which it has a lower affinity (oxacillin, cloxacillin and dicloxacillin). Indeed, during incubation time dissociation of weak antibody-antigen interactions (solution) will occur and not of strong antibody-antigen interactions (coated antigen). Free antibodies preferably will adhere to the antigens for which they have a higher affinity, resulting in lower cross-reactivities for the lower affinity antigens (Tijssen, 1985). In the biosensor the antigen-antibody interaction is measured in real-time and therefore higher cross-reactivities can be monitored for low affinity binders.

The **penicillin-specific polyclonal antibodies** were applied in an antigen ciELISA and in an antibody ciELISA (Chapter 5; Cliquet et al., submitted). For the three antisera, the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin was more sensitive in the antibody ciELISA as compared to the antigen ciELISA. The difference in sensitivity between both ELISAs could be ascribed to a combination of factors such as the use of a different competitor molecule (Choi et al., 2002), the immunogen used to produce the antibodies (Kirkley et al., 2001) and the set-up of the test system. Indeed, in the antibody ciELISA, the binding of the sample penicillins is favoured above the binding of the biotinylated penicillins due to the preincubation of sample and coated antibodies. As a result,

higher sensitivities can be measured in the antibody ciELISA as compared to the antigen ciELISA.

The **sulfonamide-specific monoclonal** mAb 3B5B10E3 was also used in an antigen ciELISA coated with PS-ova and an antibody ciELISA precoated with mouse immunoglobulin-specific antisera (Chapter 8; Cliquet et al., 2003a). Almost all tested sulfonamides were detected with the same sensitivity in both ELISA. The sensitivity of the antigen ciELISA was improved when PS-ova was used as coating antigen compared to TS-ova. Muldoon and coworkers (2000) also assessed different cross-reactivity profiles for an anti-sulfadimethoxine monoclonal when using different coating antigens. Based on their findings, it was assumed that the sensitivity of our antibody ciELISA could be improved by using the biotinylated PS-sulfonamide PS-bio instead of TS-bio. However, the absorbances obtained with PS-bio were too low to develop an assay. Our results on the penicillin-specific ELISA using polyclonals showed that an antibody ciELISA could be more sensitive as compared to the antigen ciELISA using the same antibodies. Therefore, it should be worthwhile to do some more research on the amplification of the signal or on the use of another labelled sulfonamide to develop an antibody ciELISA using our mAb 3B5B10E3. Moreover, as for the penicillin-specific mAb, it should be interesting to evaluate this mAb for its use in an optical biosensor.

Two Ag ciELISAs were developed using the **sulfonamide-specific polyclonal antibodies** pAb K3 (Chapter 9; Cliquet et al., 2003b). These ciELISAs differed from each other in the coated antigen, namely TS-ova or PS-ova. As for the mAb 3B5B10E3, no antibody ciELISA could be developed that was more sensitive than the antigen ciELISAs due to the lack of an appropriate labelled sulfonamide. The pAb K3 were obtained by immunizing rabbits with the TS-klh conjugate. Therefore higher antibody titers were obtained in the ciELISA coated with TS-ova (TS-ciELISA) than in the ciELISA coated with PS-ova (PS-ciELISA). As for the mAb 3B5B10E3, the PS-ciELISA was at least eight times more sensitive than compared to the TS-ciELISA. In the PS-ciELISA, all tested sulfonamides (sulfadiazine, -thiazole, -chloropyridazine, -methazine, -pyridine, -dimethoxine, -merazine and sulfisoxazole), except sulfisoxazole, were detected in buffer solution below 0.01 µg/ml.

In conclusion, for the mAb as well as for the pAb, our results demonstrated the importance of using an appropriate test system (ELISA, biosensor), test set-up (antigen

ciELISA, antibody ciELISA) and suitable competitor molecules (different coating antigens, labelled antigens).

10.3. Analysis of meat samples

10.3.1 Sample preparation procedures

One of the aims of this thesis was to determine whether it was possible to develop one extraction procedure for a group of antimicrobial drugs. During the development of an appropriate multi-analyte extraction procedure, it is important to take into account the distribution of the analytes in the tissues (tissue fluids, intracellular), the solubility of the analytes in the extraction buffer and the compatibility of the extract solvents with the test system (anorganic solvents are preferred for immunochemical methods). Furthermore, the extraction procedure should be kept as simple as possible to avoid loss or degradation of analyte during handling and to reduce the time for sample analysis.

In literature, no simple procedure was described for the extraction of all penicillins or all sulfonamides in meat samples for analysis in an immunochemical assay. Therefore, we developed simple sample preparation procedures for meat samples containing penicillins or sulfonamides. Recently, simple extraction procedures were reported for the analysis of penicillins and sulfonamides containing meat samples using the Parallux™ assay (Okerman et al., 2003) and the Biacore® biosensor (McGrath et al., 2004).

Usually, meat samples are minced using a conventional kitchen mixer before homogenization with an appropriate extraction buffer (McCracken et al., 2000). However, this way of working produced high background signals in our penicillin-specific ELISAs. Therefore, the tissue samples were cut into small pieces with a knife instead of minced with a mixer (Chapter 7). Working with minced tissues implied a centrifugation step. The extracts were subsequently filtered and adjusted to pH 7. This rather simple extraction procedure still took too much time. Therefore, we investigated the possibility of using sample fluids instead of tissues. Penicillins are present in the intercellular fluid of tissues (Divers, 1996). The use of tissue fluid simplified the sample preparation. Before analysis in ELISA, the fluids were treated with kaolin to reduce background signals in ELISA by removing disturbing fat or other matrix components (Van den Broeck et al, 1999). For the analysis of kidneys using the Parallux™ assay, tissue samples are homogenized with the extraction buffer using a

Stomacher, whereafter a centrifugation step has to be performed to obtain the extraction fluid (Okerman et al., 2003). Our procedure only required an eppendorf centrifuge.

Mostly, organic solvents are used for the extraction of sulfonamides (Guggisberg et al., 1992). However, these solvents are not very compatible with immunochemical methods. Therefore, we preferred to use an anorganic buffer (Chapter 9; Cliquet et al., 2003b). A carbonate/bicarbonate buffer (pH 10) was chosen in which sulfonamides are highly soluble (Budavari, 1988). Differences in homogenizing techniques (high-speed mixer (ultraturax) versus vortex) and the effect of kaolin were evaluated. The best extraction procedure was the simplest method, using a vortex mixer as homogenizer and no kaolin treatment. A similar procedure is applied when analyzing samples using the Biacore® biosensor Qflex assay (McGrath et al., 2004).

10.3.2. Validation of the ELISAs

In order to determine if it was possible to make a screening assay using group-specific antibodies, not only antibodies, assays and extraction procedures had to be developed, but tests had to be properly evaluated. Indeed, any method or combination of methods may only be used for screening or confirmatory purposes if it can be proven that they fulfill the requirements established in the Commission Decision 2002/657/EC. For screening purposes, only those methods that are validated and have a false-compliant rate lower than 5% at the MRL are allowed. Validation of a method means demonstrating that the method complies with the criteria applicable for the relevant performance characteristics. For a qualitative screening method, the detection capability (CCβ), the selectivity/specificity and the applicability/ruggedness/stability must be demonstrated. When a screening assay will be used quantitatively, the precision must also be determined (Anonymous, 2002).

Some of the relevant performance characteristics (the specificity, sensitivity, repeatability, decision limit (CCα) en detection capability (CCβ)) were determined for the penicillin-specific antibody ciELISA with pAb K2 and for the sulfonamide-specific antigen ciELISA with pAb K3 (Chapter 7 and 9).

The detection capability and decision limit were determined according to the Commission Decision 2002/657/EC, for amoxicillin (CCα =9 ppb; CCβ =19 ppb) and sulfachloropyridazine (CCα = 9.4 ppb, CCβ = 12.9 ppb) in porcine kidneys. These ELISAs are multi-analyte assays for penicillins and sulfonamides, respectively. Their sensitivity is not

the same for all analytes. Amoxicillin was chosen as standard analyte in the penicillin ELISA. The detection capability and decision limit were established for that standard penicillin. However, the identity of a penicillin in a sample is not known during screening. Therefore, decision based on CCβ (non-compliant if the concentration is higher than CCβ) will not necessarily give certainty about the false compliant rate of the ELISA. For the sulfonamide-specific ELISA, CCα and CCβ were determined for sulfachloropyridazine because incurred samples were available for that sulfonamide. Sulfachloropyridazine cannot be used as reference sulfonamide because the sensitivity of this assay is much higher for that sulfonamide than for most of the tested sulfonamides (Chapter 9). The decision limit and detection capability for amoxicillin and sulfachloropyridazine are rather giving information about the precision of the measurements of the penicillin-specific and sulfonamide-specific ELISA, respectively. Since a sample is considered non-compliant when the concentration is higher than the CCβ, compliant when lower than CCα and suspect when between CCα and CCβ, the range between CCα and CCβ should be as narrow as possible. This is obtained when the repeatability of the ELISA is high (small variations between repeated analyses). The coefficient of variation should not exceed 15 % (Crabbe, 2002). This requirement was fulfilled for both ELISAs (Chapter 7 and 9). Compared to the penicillin-specific ELISA (CCα =9 ppb; CCβ =19 ppb), the sulfonamide-specific ELISA is thus more precise (CCα = 9.4 ppb, CCβ = 12.9 ppb).

The ideal group-specific assay would detect all members of the group with the same sensitivity (cross-reactivities 100%). At that moment, decision based on CCβ can give a correct idea about the compliance of a sample.

To determine the detection capability of the ELISA in accordance to the Commission Decision 2002/657/EC, fortified samples containing penicillin at the MRL were used. Fortified samples are more appropriate than samples obtained from in vivo experiments because it is difficult to predict the concentration that will be obtained in the tissues after administration of the drug to animals. On the other hand, attention must be taken when validating a method based on fortified samples. An extraction protocol optimized using fortified samples will not necessarily be efficient for incurred samples. The efficiency of the extraction procedure to extract analytes from the tissue matrix cannot be determined using fortified samples because the interaction between analyte and the sample matrix is different than for incurred samples (Mc Cracken et al., 2000). Therefore, the analysis of incurred

samples in the ELISAs was compared to the analysis of the same samples using other qualitative or quantitative methods.

Because the antibody ciELISA with pAb K2 is designed as a qualitative screening assay, the efficiency of the extraction procedure and analysis of samples was investigated by analyzing 15 incurred samples and comparing the results with these obtained using two other qualitative screening assays, the New Belgian Kidney Test (NBKT) and the Parallux™ assay. Almost 100 % correlation (r^2 = 0.94) was found between the Parallux™ and the ELISA. Because the incurred samples came from animals with unknown treatment, and because not only penicillins but also other substances with antimicrobial activity are detected in the NBKT, a positive result in NBKT indicated the presence of one or more antimicrobial substances and thus not only penicillins. This could be the reason why two samples were tested positive in the NBKT, but negative in the Parallux™ and the ELISA.

For the development of an appropriate extraction procedure for the sulfonamide-specific ELISA, fortified porcine tissue samples were used. Two procedures were selected based on the recovery and repeatability (procedure 3 and 5). The comparison of the analysis of kidneys and livers from pigs experimentally treated with sulfachloropyridazine in the PS-ciELISA with the analysis of the same samples using LC-MS/MS method, allowed us to select one of both extraction procedure. The comparative study demonstrated that extraction procedure 3, using a vortex mixer as homogeniser and no kaolin treatment, was more suitable than procedure 5, using a vortex mixer and kaolin treatment, for the extraction of sulfachloropyridazine from both kidney and liver tissues (Chapter 9).

10.4. Main conclusions and future perspectives

In this study, the development of group-specific screening assays using antibodies was investigated. The hope was to find a general strategy for the development of group-specific assays based on the results obtained for penicillins and sulfonamides.

The biggest part of the research was the development of group-specific antibodies. Results showed that is difficult to build up a general strategy for making an optimal immunogen, because the immunogenicity of an antigen will not necessarily guarantee the production of suitable antibodies. The screening procedure for selecting antibodies is as important.

It was possible to develop antibodies that recognize a broad range of antimicrobials of the same group and, although nothing is absolute, following parameters are thought to be critical for the development of group-specific antibodies:

- The development of an appropriate immunogen, taking into account that the potential of inducing a high immune response is not necessarily the most important selection parameter.
- The immunization procedure (mice) including a final intravenous and intraperitoneal boost injection.
- The procedure for the screening of sera and hybridomas.

Using the monoclonal and polyclonal penicillin- and sulfonamide-specific antibodies, different test systems were developed, namely antigen ciELISAs, antibody ciELISAs and biosensor assays. Thus, it was possible to develop group-specific assays using our group-specific antibodies. The choice of the test system influenced the sensitivity and specificity of the antibodies. Therefore, when developing a detection system using antibodies, the screening assay should resemble the final test as much as possible. Results showed that the detection of analytes using polyclonal antibodies is in general more sensitive than using monoclonals.

For the analysis of samples, appropriate extraction procedures were developed for the group of penicillins and for the group of sulfonamides, respectively. Most important factors are the extraction buffer and the sample handling, which should be kept as minimal as possible.

A method or combination of methods may only be used for screening if it can be proven that they fulfill the relevant requirements established in the Commission Decision 2002/657/EC. This validation is performed using fortified samples. It is advisable, however, to compare the analysis of incurred samples using a reference method. An extraction procedure developed using fortified samples will not necessarily be efficient for incurred samples because the interaction of the analyte with the sample matrix can be different. The comparative studies of the analysis of incurred tissues using our ELISAs and using a commercial immunochemical assay (penicillins) or a physico-chemical method (sulfonamides) demonstrated the suitability of the ELISAs and the corresponding extraction procedure.

Summary

Microbiological inhibition assays are mostly applied for the screening of food products of animal origin. These tests have a broad-spectrum specificity and are therefore useful for screening purposes. A sample with a positive result in the microbiological test must be confirmed with a physico-chemical method. Such techniques can be very reliable and precise, but they are expensive and labour-intensive. It is therefore advisable to avoid false non-compliant results during screening. Moreover, before analysing with a physico-chemical method, it would be advisable to determine to which family of antibiotics the unknown compound in the presumed non-conform sample belongs, because the broad-spectrum microbiological assays do not discriminate between different families of antibiotics. The aim of this study was to investigate the possibility of developing such group-specific screening tests using antibodies specific for penicillins or sulfonamides. Such group-specific antibodies should allow the development of an immunochemical assay, like the enzyme-linked immunosorbent assay (ELISA). In combination with an appropriate extraction procedure, the ELISAs can then be applied for the detection of penicillins and sulfonamides, respectively, in meat samples.

Chapter 1 gives an overview of the principles of immunoassays. Because such assays are based on the interaction between antigen and antibody, the physicochemical properties of the interaction and the influencing factors are reviewed. The nature of immunogens, antigens or haptens, is discussed. The production of antibodies is explained and the properties of monoclonal and polyclonal antibodies are compared. An overview is given of immunological methods applied in this work, namely ELISA and the optical biosensor, in which detection is based on surface plasmon resonance.

Chapter 2 and 3 review the application of penicillins and sulfonamides, respectively, in veterinary medicine. Their physicochemical and biological properties, and their metabolisation are discussed. Furthermore, the consequences of the abuse of penicillins and sulfonamides in veterinary husbandry are mentioned. In addition, an overview is given on the current state on detection methods, microbiological inhibition assays, receptor assays and immunoassays.

Chapters 4 to 9 present the experimental work of this research. The aim of the study was to answer to following questions:

1. Is it possible to develop antibodies recognizing a group of antimicrobials?

2. Can a screening assay be developed using group-specific antibodies?
3. Is it possible to develop one extraction procedure for a group of antimicrobials?

The development of penicillin-specific monoclonal antibodies (mAb) is discussed in chapter 4. Several procedures were evaluated to obtain monoclonal antibodies specific for the common structure of penicillins. Ampicillin was coupled to different carrier-proteins (bovine serum albumin, chicken ovalbumin and thyroglobulin) to render it immunogenic. Hereto, different coupling methods were compared, namely two methods using a cross-linker (glutaraldehyde or a succinimide ester), one carbodiimide-mediated coupling method and one method without a cross-linker or mediator molecule (physiological binding). Mice were immunised with the conjugates intraperitoneally, intravenously or in the footpad. A screening-ELISA was developed to detect anti-ampicillin antibodies in sera. Specificity and affinity of the antibodies were demonstrated by inhibiting their binding to coated ampicillin-protein conjugate with a 10 mM solution of ampicillin. No difference in the obtained hybridoma could be observed using electrofusion or PEG-mediated fusion. For the production of the monoclonals, an intraveneous final boost gave antibodies with better specificity and affinity than an intraperitoneal final booster injection. Two anti-ampicillin monoclonals (mAb 19C9 and mAb 9H3) were selected that cross-reacts with penicillin G, oxacillin, cloxacillin, dicloxacillin and carbenicillin, and not with cephalosporins (cephadrin, cephalexin, cefaclor), clavulanic acid, sulfanilamide, chloramphenicol, neomycin and streptomycin, and were therefore interesting for trying to develop a penicillin-specific ELISA.

The production of penicillin-specific polyclonal rabbit antibodies (pAb) and the application of these antibodies in an ELISA are described in chapter 5. The polyclonals were obtained after immunization of three rabbits (K2, K6 and K8) with physiological ampicillin- and benzylpenicillin-protein conjugates (pAb K2) or with physiological ampicillin-, benzylpenicillin-, oxacillin and dicloxacillin-protein conjugates (pAb K6 and pAb K8). The broad-specificity of the antisera induced by physiologic penicillin-protein conjugates was improved by alternately immunizing the animals with conjugates containing different penicillins as hapten. With each of the polyclonals, an antigen and an antibody competitive inhibition (ci) ELISA was developed. For the three antisera, the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin was more sensitive in the antibody ciELISA as compared to the antigen ciELISA. However, the detection of all penicillins in buffer solutions below the MRL in the antibody ciELISA was only achieved

when the penicillins were hydrolysed with Penicillinase I. No cross-reactions were observed for cephadrin, cefalexin, cefazolin, clavulanic acid, sulfanilamide or chloramphenicol.

In chapter 6, the monoclonals 19C9 and 9H3 and polyclonals K2 are compared for their use in ELISA and in the BIAcore™ optical biosensor. In the ELISA, an ampicillin-protein conjugate was used as coating molecule whereas for the biosensor assay, ampicillin was directly immobilized on a CM5 chip. With both monoclonal antibodies and in both testsystems, ampicillin, amoxicillin and benzylpenicillin were better recognized than oxacillin, cloxacillin and dicloxacillin. Because the reproducibility was better in the biosensor (CV = 1.6 %) than in the ELISA (CV = 8.9 %), the limit of detection for ampicillin in a buffer solution using mAb 19C9 was lower in the biosensor (46 ng/ml) than in the ELISA (356 ng/ml). Ampicillin could thus be detected below the MRL (50 ng/ml) in the biosensor assay but not in the ELISA.

Both the ELISA and biosensor assay using pAb K2 were more sensitive as compared to the assays with the monoclonals. Indeed, the ELISA using pAb K2 allowed the detection of all tested penicillins below the MRL, whereas in the biosensor assay, ampicillin was detected with an IC50 of 10 ng/ml which is lower as using mAb 19C9 (IC50 = 524 ng/ml). In contrast to the binding of the monoclonals, no spontaneous dissociation was observed after injection of the polyclonal antibodies in the biosensor. Whereas the monoclonals were completely removed from the sensor surface using ampicillin in buffer solution (500 µg/ml) as regeneration solution, stronger conditions (0.1 M NaOH containing 20% acetonitril) were necessary for removing the pAb. Probably the detection of ampicillin in the biosensor assay using pAb K2 can still be improved by hydrolysis of the ampicillin using Penicillinase I before analysis.

With the monoclonals (mAb 19C9 and mAb 9H3) as well as with the polyclonal antibodies (pAb K2, pAb K6 and pAb K8), an antigen ciELISA was developed for the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin in buffer solutions (Chapter 6). However, the antigen ciELISAs were not able to detect all tested penicillins below the MRL (50 µg/kg). Ampicillin was only detected sensitive enough in the antigen ciELISA with the polyclonals (LOD = 6 ng/ml with pAb K2; LOD = 20 ng/ml with pAb K6 and pAb K8) and amoxicillin in the antigen ciELISA with mAb 19C9 (LOD = 18 ng/ml). With the polyclonals, but not with the monoclonals, an antibody ciELISA could be developed that was ten times more sensitive than the antigen ciELISA. Therefore only the ELISAs using pAb K2 were chosen to assess the possibility of analysing porcine tissues according to the European requirements for screening tests used for the inspection of food

derived from treated animals (Chapter 7). Some of the relevant performance characteristics (specificity, sensitivity, repeatability, decision limit (CCα) and detection capability (CCβ)) for the validation are discussed. Results demonstrated that the precision of the antibody ciELISA is higher than this of the antigen ciELISA: the range between CCα and CCβ is smaller for the analysis of kidney samples in the antibody ciELISA (9-19 ng/ml) as compared to the antigen ciELISA (21-69 ng/ml). Since a sample is considered non-compliant when the concentration is higher than the CCβ, compliant when lower than CCα and suspected when between CCα and CCβ, the range between CCα and CCβ should be as narrow as possible. The suitability of the antibody ciELISA and of the used extraction procedure for screening purposes was demonstrated by comparing the analysis of the ELISA results for incurred samples with these of a commercial immunochemical method, the Parallux™ assay. Almost 100 % correlation ($r^2 = 0.94$) was found.

Chapter 8 reports the different attempts to obtain monoclonal antibodies specific for the common structure of sulfonamides. In a first approach, sulfanilamide was linked to albumins using glutaraldehyde or a succinimide ester as cross-linker. No or a weak immune response was induced after immunization of mice with these conjugates. High antibody titers were obtained with conjugates of sulfanilamide linked to albumins or casein with a diazotation reaction. However, the antibodies were only highly specific for the bound sulfanilamide molecule. In a second approach, sulfonamide-protein conjugates were used in which the sulfonamide molecule was linked at its side chain, leaving the common structure of sulfonamides unchanged. Hereto, three sulfonamide derivatives (N-sulfanyl-4-aminobenzoic acid (S), N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS) and N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS)), previously described in literature, containing a carboxyl group in their side chain were synthesized and subsequently linked to proteins using a carbodiimide-mediated reaction. Immunization with the S-conjugates led to high antibody titers, but the antibodies were only highly specific for the bound S-molecule. Group-specific antibodies were obtained after immunization with the PS- and TS-conjugates. It had been described that immunization with PS-conjugates led to the recognition of other sulfonamides (sulfamethazine, -merazine, -diazine and –dimethoxine) that are not well recognized by antibodies induced after immunization with TS-conjugates. Therefore, we tried to guide the immune response in the direction of recognition of the common structure of sulfonamides by immunizing the animals alternately with PS- and TS-conjugates. The polyclonal antibodies of the mice had indeed a broader specificity, but the

specificity of the monoclonals obtained after fusion experiments was not influenced. Immunization with TS-conjugates seemed sufficient to obtain sulfonamide-specific monoclonal antibodies. With the best monoclonal (mAb 3B5B10E3) two competitive inhibition (ci) ELISAs were developed: one coated with antigen and the other coated with the monoclonal antibody. Sulfadiazine, –dimethoxine, -thiazole, -pyridine and -methoxazole were detected in both ELISAs at their MRL-value (100 ng/ml) in buffer solution. Sulfadiazine, sulfathiazole and sulfamethoxazole could even be detected at 10 ng/ml. Cross-reactivities were noticed for diuretics (furosemide, acetazolamide, hydrochlorothiazide, bumetanide; IC50 >10 µg/ml), but not for thiamphenicol, florphenicol, lidocaine and *p*-aminobenzoic acid. Since the diuretics only have the $–SO_2NH-$ group in common with sulfonamides, this indicates that the epitope recognized by the monoclonal contains the $–SO_2NH-$ group.

In chapter 9, the development of an ELISA using sulfonamide-specific polyclonal rabbit antibodies (pAb K3) is described. The antibodies were obtained after immunization with the TS- keyhole limpet hemocyanin (TS-klh) immunogen. Using these antibodies, two sulfonamide-specific ELISAs were developed differing in coating antigen: TS-ovalbumin (TS-ova) and PS-ovalbumin (PS-ova). The detection of sulfamethazine, sulfamerazine, sulfadimethoxine, sulfadiazine, sulfathiazole, sulfapyridine, sulfachloropyridazine and sulfisoxazole in buffer was analysed. Higher antibody titers were obtained in the ciELISA coated with TS-ova (TS-ciELISA) than in the ciELISA coated with PS-ova (PS-ciELISA), but the detection of sulfonamides was more sensitive in the PS-ciELISA, allowing the detection of all tested sulfonamides at the MRL-value (100 ng/ml). Cross-reactivities of pAb K3 were noticed for para-aminobenzoic acid (IC50 >10 µg/ml), but not for diuretics (furosemide, acetazolamide, hydrochlorothiazide, bumetanide), thiamphenicol, florphenicol and lidocaine. Para-aminobenzoic acid contains the common sulfonamide *p*-aminobenzoyl ring. This indicates that the epitope recognized by the polyclonals contains *p*-aminobenzoyl ring. Substitution of the sulfonamide-group on the common *p*-aminobenzoyl ring favors the binding of the polyclonals since most sulfonamides are recognized with higher sensitivity (IC50 <1 µg/ml) than *p*-aminobenzoic acid (IC50 >10 µg/ml).

In a subsequent step, an extraction procedure was developed for the detection of sulfonamides, in muscles, kidney, liver and fat, by both ELISAs using sulfachloropyridazine as model. As extraction buffer a carbonate/bicarbonate buffer (pH 10) was chosen in which sulfonamides are highly soluble. Differences in homogenizing techniques (high-speed mixer (ultraturax) versus vortex) and the effect of kaolin (hydrated aluminum silicate) treatment, to

diminish the background signal in ELISA, were evaluated. The best extraction procedure was the one using a vortex mixer as homogenizer and no kaolin treatment. Sulfachloropyridazine was easily detected at the MRL in all tissues. The decision limit and detection capability for sulfachloropyridazine in porcine kidneys were determined (CCα = 9.4 ng/ml, CCβ = 12.9 ng/ml). Acceptable correlations were found between the PS-ciELISA and LC-MS/MS for the analysis of incurred porcine liver and kidney samples (r^2 = 0.88 and 0.77 resp.).

A general discussion and conclusions are given in Part V of this thesis. The results of this thesis show that it is possible to develop antibodies recognizing a group of antimicrobials. To obtain such antibodies, an appropriate immunogen for induction of group-specific antibodies is very important. Other conclusions that can be drawn are that alternately injecting different immunogens enhances the polyclonal response and that a final intravenous booster immunization enhances hybridoma production. However, neither the immunogen nor the immunization procedure can guarantee the production of suitable antibodies. The test for selecting the antibodies is as important and should resemble as close as possible the test that will be developed for detecting the analytes in food of animal origin.

Using the monoclonal and polyclonal penicillin- and sulfonamide-specific antibodies, different test systems were developed, namely antigen ciELISAs, antibody ciELISAs and biosensor assays. Thus, it is possible to develop group-specific assays using our group-specific antibodies. It was demonstrated that the choice of the test system influenced the sensitivity and specificity of the antibodies. In our research the detection of analytes using polyclonal antibodies was more sensitive than using monoclonals.

For analysing tissue samples, an appropriate simple extraction procedure had to be developed. This was achieved for penicillins as well as for sulfonamides. It is thus possible to develop one extraction procedure for a group of antimicrobials. Very important factors are the extraction buffer and the sample handling, which should be kept as minimal as possible.

Any method or combination of methods may only be used for screening or confirmatory purposes if it can be proven that they fulfill the relevant requirements established in the Commission Decision 2002/657/EC. For a qualitative screening method, the detection capability (CCβ), the selectivity/specificity and the applicability/ruggedness/stability must be demonstrated. When a screening assay will be used quantitatively, the precision must also be determined. The validation is performed using fortified samples. It is advisable, however, to compare the analysis of incurred samples with the analysis using a reference method. An extraction procedure developed using fortified

samples will not necessarily be efficient for incurred samples because the interaction of the analyte with the sample matrix can be different in fortified samples as compared to incurred samples. The comparative studies of the analysis of incurred tissues using our ELISAs and using a commercial immunochemical assay, in case of penicillins, or a physico-chemical method, in case of sulfonamides, demonstrated the suitability of our ELISAs and of the corresponding extraction procedures.

Samenvatting

Microbiologische inhibitietesten worden meestal gebruikt voor het screenen van voedingswaren van dierlijke oorsprong. Deze testen hebben een brede spectrumspecificiteit en zijn daarom nuttig voor screening. Wanneer een staal positief (niet-conform) bevonden wordt met een microbiologische test, dan moet dit resultaat bevestigd worden met chemische analysemethoden. Deze methoden kunnen heel betrouwbaar en nauwkeurig zijn, maar anderzijds zijn ze arbeidsintensief en duur. Het is daarom aangeraden om tijdens de screening foutieve niet-conforme resultaten te vermijden. Bovendien moet men eerst bepalen tot welke groep antibiotica het onbekende residu in het niet-conforme staal behoort, zodat de meest geschikte confirmatiemethode kan gekozen worden. Breed-spectrum microbiologische testen maken geen onderscheid tussen de verschillende antibioticafamilies. Het doel van deze studie was om na te gaan of groepsspecifieke antistoffen kunnen worden ontwikkeld voor penicillines en voor sulfonamiden. Met zulke groepsspecifieke antistoffen zou het vervolgens mogelijk moeten zijn om een immunochemische test te ontwikkelen, zoals de enzyme-linked immunosorbent assay (ELISA). Zo'n test kan dan gebruikt worden voor de groepsidentificatie van een vermoedelijk niet-conform staal. In combinatie met een geschikte extractieprocedure zouden deze ELISA's dan kunnen gebruikt worden voor de detectie van penicillines en sulfonamiden in vlees.

In hoofdstuk 1 wordt een overzicht gegeven van de principes van immunochemische testen. Omdat deze testen gebaseerd zijn op de interactie tussen antistof en antigeen, worden de fysicochemische eigenschappen van de interactie en de beïnvloedende factoren besproken. De productie van antistoffen wordt behandeld waarbij een vergelijking gemaakt wordt tussen monoklonalen en polyklonalen. Vervolgens wordt ook een overzicht gegeven van de immunologische testen gebruikt in deze thesis, nl de ELISA en de optische biosensor op basis van oppervlakte plasmonresonantie.

In hoofdstuk 2 en 3 wordt het gebruik van penicillines en sulfonamiden in de diergeneeskunde besproken. Hun fysicochemische en biologische eigenschappen, alsook hun metabolisatie worden behandeld. Verder worden de gevolgen van het overmatige gebruik van penicillines en sulfonamiden toegelicht. Tenslotte wordt een overzicht gegeven van de huidige stand van zaken betreffende de beschikbare microbiologische inhibitietesten, receptortesten en immunochemische testen.

In hoofdstuk 4 tot en met 9 wordt het experimentele werk van dit onderzoek weergegeven. Deze thesis had tot doel volgende vragen te beantwoorden:

1. Is het mogelijk om antistoffen te ontwikkelen specifiek voor een groep antimicrobiële stoffen?
2. Kan een screeningstest ontwikkeld worden met zulke groepsspecifieke antistoffen?
3. Is het mogelijk om een extractieprocedure te ontwikkelen voor een groep antimicrobiële stoffen?

De ontwikkeling van penicilline-specifieke monoklonale antistoffen wordt besproken in hoofdstuk 4. Verschillende procedures werden geëvalueerd teneinde monoklonalen te bekomen die specifiek zijn voor de gemeenschappelijke structuur van penicillines. Hiertoe moesten penicillines immunogeen gemaakt worden. Teneinde ampicilline immunogeen te maken, werd het gekoppeld aan verschillende drager-eiwitten (bovien albumine, kippenalbumine and thyroglobuline). Hierbij werden verschillende koppelingsmethoden vergeleken: twee methoden gebruikmakend van een crosslinkermolecule (glutaaraldehyde of een succinimide ester), een carbodiimide-gemediëerde koppeling en een methode zonder crosslinkermolecule of mediator (fysiologische binding). Muizen werden intraperitoneaal, intraveneus of in de voetzool geïmmunizeerd. Een screenings-ELISA werd ontwikkeld voor de detectie van de anti-ampicilline antistoffen in sera. De specificiteit en affiniteit van de antistoffen werd getest door de inhibitie van hun binding aan de gecoate ampicilline-eiwitconjugaten na te gaan in aanwezigheid van ampicilline (10 mM in bufferoplossing). Voor de productie van monoklonalen werd geen verschil waargenomen in het aantal hybridomen bekomen na elektrofusies en PEG-gemediëerde fusies. Antistoffen met een hogere specificiteit en affiniteit werden bekomen na een intraveneuze finale injectie in vergelijking met een intraperitoneale injectie. Twee anti-ampicilline monoklonalen (mAb 19C9 en mAb 9H3) werden geselecteerd die kruisreageren met benzylpenicilline, oxacilline, cloxacilline, dicloxacilline en carbenicillin, en niet met cefalosporinen (cefadrine, cefalexine, cefaclor), clavulaanzuur, sulfanilamide, chloramphenicol, neomycin en streptomycin. Bijgevolg werden deze monoklonalen interessant bevonden voor de ontwikkeling van een penicilline-specifieke ELISA.

De productie van penicilline-specifieke polyklonale konijnenantistoffen (pAb) en het gebruik van deze antistoffen in een ELISA wordt beschreven in hoofdstuk 5. De polyklonalen werden bekomen na immunizatie van 3 konijnen (K2, K6 en K8) met fysiologische

ampicilline- en benzylpenicilline-eiwitconjugaten (pAb K2) of met fysiologische ampicilline-, benzylpenicilline-, oxacilline and dicloxacilline-eiwitconjugaten (pAb K6 en pAb K8). De groepsspecificiteit van de antisera geïnduceerd met fysiologische penicilline-eiwitconjugaten kon worden verbeterd door de dieren afwisselend te immunizeren met conjugaten die een verschillend hapteen bevatten. Met elk polyklonaal werden zowel een met antigeen gecoate competitieve inhibitie ELISA (antigeen ciELISA) als een met antistof gecoate competitieve inhibitie ELISA (antistof ciELISA) ontwikkeld. Voor alle drie de antisera was de detectie van ampicilline, amoxicilline, benzylpenicilline, oxacilline, cloxacilline and dicloxacilline gevoeliger in de antistof ciELISA dan in de antigeen ciELISA, doch de detectie van alle penicillines in bufferoplossing onder de MRL was enkel mogelijk in de antistof ciELISA als de penicillines voorafgaandelijk gehydrolyseerd werden met Penicillinase I. Er werden geen kruisreacties waargenomen voor cefadrine, cefalexine, cefazoline, clavulaanzuur, sulfanilamide en chloramphenicol.

In hoofdstuk 6 wordt het gebruik vergeleken van de monoklonalen 19C9 en 9H3, en de polyclonaal K2 in ELISA en in de BIAcore™ optische biosensor. In de ELISA werd een ampicilline-eiwitconjugaat gebruikt als coatingsmolecule terwijl in de biosensortest ampicilline rechtstreeks geïmmobiliseerd werd op een CM5 chip. Met beide monoklonale antistoffen en in beide testsystemen, werden ampicilline, amoxicilline en benzylpenicilline beter herkend dan oxacilline, cloxacilline en dicloxacilline. Omdat bij gebruik van mAb 19C9, de herhaalbaarheid hoger was in de biosensortest (CV = 1.6 %) dan in de ELISA (CV = 8.9 %), was de detectielimiet voor ampicilline in bufferoplossing lager in de biosensor (46 ng/ml) dan in de ELISA (356 ng/ml). Ampicilline kon bijgevolg gedetecteerd worden aan de MRL (50 ng/ml) in de biosensortest maar niet in de ELISA.

Zowel de ELISA als de biosensortest gebruikmakend van de polyklonale antistoffen was gevoeliger dan de testen gebruikmakend van de monoklonalen. Met de polyklonalen kunnen alle geteste penicillines in de ELISA en ampicilline in de biosensortest tot onder de MRL gedetecteerd worden. In de biosensortest werd, in tegenstelling tot de binding van de monoklonalen, geen spontane dissociatie waargenomen na injectie van de polyklonale antistoffen in de biosensor. De monoklonalen konden gemakkelijk van het sensoroppervlak verwijderd worden met een overmaat ampicilline (500 µg/ml) als regeneratievloeistof. Voor de polyklonalen waren echter strengere regeneratiecondities nodig (0.1 M NaOH + 20% acetonitril). De detectie van ampicilline in de biosensortest gebruikmakend van de pAb K2 kan waarschijnlijk nog verbeterd worden door de stalen vooraf te behandelen met Penicillinase I.

Zowel met de monoklonalen (mAb 19C9 en mAb 9H3) als met de polyklonale antistoffen (pAb K2, pAb K6 en pAb K8) werd een antigeen ciELISA ontwikkeld voor de detectie van ampicilline, amoxicilline, benzylpenicilline, oxacilline, cloxacilline en dicloxacilline in bufferoplossing (Hoofdstuk 5 en 6). De antigeen ciELISA's waren echter niet in staat om alle geteste penicillines te detecteren tot onder de MRL (50 µg/kg). Ampicilline werd enkel gevoelig genoeg gedetecteerd in de antigeen ciELISA met de polyklonalen (LOD = 6 ng/ml met pAb K2; LOD = 20 ng/ml met pAb K6 en pAb K8) en amoxicilline in de antigeen ciELISA met mAb 19C9 (LOD = 18 ng/ml). Met de polyklonalen, maar niet met de monoklonalen, werd een antistof ciELISA ontwikkeld die tienmaal gevoeliger was dan de antigeen ciELISA. Daarom werden enkel de ELISA's gebruikmakend van pAb K2 gekozen om na te gaan of varkensweefsels geanalyseerd kunnen worden overeenkomstig de Europese richtlijn voor het gebruik van screeningstesten voor de inspectie van voedingswaren van dierlijke oorsprong (Hoofdstuk 7). Een aantal relevante karakteristieken (specificiteit, gevoeligheid, herhaalbaarheid, beslissingsgrens (CCα) en detectievermogen (CCβ)) voor de validatie worden besproken. De resultaten tonen aan dat de nauwkeurigheid van de antistof ciELISA hoger is dan deze van de antigeen ciELISA: de spreiding tussen CCα en CCβ is kleiner voor de analyse van nierweefsels in de antistof ciELISA (9-19 ng/ml) dan in de antigeen ciELISA (21-69 ng/ml). Immers, een staal is niet-conform wanneer de concentratie hoger is dan CCβ, conform wanneer deze lager is dan CCα en verdacht wanneer de concentratie tussen CCα en CCβ ligt. Dit betekent dat de spreiding tussen CCα en CCβ liefst zo klein mogelijk moet zijn. Bovendien werd de geschiktheid van de antistof ciELISA en de gebruikte extractieprocedure voor het screenen van varkensweefsels aangetoond door de ELISA resultaten bekomen voor praktijkstalen te vergelijken met de resultaten van de analyse van dezelfde stalen met een commerciële immunochemische methode, de Parallux™ test. Er werd bijna 100 % overeenkomst waargenomen tussen de resultaten van de ELISA en deze van de Parallux™ test ($r^2 = 0.94$).

In hoofdstuk 8 worden de verschillende strategieën beschreven om monoklonale antistoffen te bekomen specifiek voor de gemeenschappelijke sulfonamidenstructuur. Een eerste strategie bestond erin sulfanilamide te koppelen aan albumines met behulp van glutaaraldehyde of een succinimide ester als crosslinkermolecule. Immunizatie van muizen met deze conjugaten induceerde geen of zwakke immuunresponsen. Hoge antistoffentiters werden bekomen na immunizatie met sulfanilamide gekoppeld aan albumines of caseïne via de diazotatiereactie. Deze antistoffen waren echter enkel heel specifiek voor de gebonden

sulfanilamide molecule. In een tweede strategie werden sulfonamideneiwitconjugaten gebruikt waarin de sulfonamidenmolecule via zijn zijketen gekoppeld was zodat de gemeenschappelijke sulfonamidenstructuur ongewijzigd bleef. Hiervoor werden drie sulfonamidenderivaten (N-sulfanyl-4-aminobenzoic acid (S), N1-[4-methyl-5-[2-4-carboxyethyl-1-hydroxyphenyl)]-azo-2-pyridyl]-sulfanilamide (PS) en N1-[4-(carboxymethyl)-2-thiazolyl]sulfanilamide (TS)), gesynthetiseerd. Deze derivaten bevatten een carboxylgroup in hun zijketen waarmee ze aan eiwitten kunnen worden gebonden via een carbodiimide-gemediëerde reactie. Immunizatie met de S-eiwitconjugaten resulteerde in hoge antistoffentiters. De antistoffen waren echter zeer specifiek voor de gebonden S-molecule. Groepsspecifieke antistoffen werden wel bekomen na immunizatie met de PS- en TS-eiwitconjugaten. Het was reeds beschreven in de literatuur dat immunizatie met PS-eiwitconjugaten leidde tot de herkenning van andere sulfonamiden (sulfamethazine, -merazine, -diazine en –dimethoxine) die niet goed herkend werden door antistoffen geïnduceerd na immunizatie met TS-eiwitconjugaten. Bijgevolg werd geprobeerd de immuunrespons te richten naar herkenning van de gemeenschappelijke sulfonamidenstructuur door de dieren afwisselend te immunizeren met PS- en TS-eiwitconjugaten. De polyklonale antistoffen van de muizen hadden inderdaad een bredere specificiteit, maar de monoklonalen bekomen na fusie werden hierdoor niet beïnvloed. Immunizatie met TS-eiwitconjugaten bleek voldoende om sulfonamidenspecifieke monoklonale antistoffen te bekomen. Met het beste monoklonaal (mAb 3B5B10E3) werden twee competitieve inhibitie (ci) ELISA's ontwikkeld: één gecoat met antigeen en de andere gecoat met de monoklonale antistof. Sulfadiazine, –dimethoxine, -thiazole, -pyridine en -methoxazole werden in beide ELISA's in bufferoplossingen gedetecteerd aan de MRL (100 ng/ml). Sulfadiazine, sulfathiazole en sulfamethoxazole konden zelfs tot 10 ng/ml gedetecteerd worden. Kruisreacties werden enkel waargenomen voor diuretica (furosemide, acetazolamide, hydrochlorothiazide, bumetanide; IC50 > 10 µg/ml), maar niet voor thiamfenicol, florfenicol, lidocaine en *p*-aminobenzoëzuur. Daar de diuretica enkel de $–SO_2NH-$ groep gemeenschappelijk hebben met de sulfonamiden, betekent dit dat het epitoop dat door het monoklonaal herkend wordt de $–SO_2NH-$ groep bevat.

In hoofdstuk 9 wordt de ontwikkeling van een ELISA gebruikmakend van de sulfonamidenspecifieke polyklonale konijnenantistoffen (pAb K3) beschreven. De antistoffen werden bekomen na immunizatie met het TS-Keyhole limpet hemocyanine (TS-klh)

immunogeen. Met deze antistoffen werden twee sulfonamidenspecifieke ELISA's ontwikkeld die van elkaar verschillen in de coatingsmolecule, namelijk TS-ovalbumine (TS-ova) in de ene en PS-ovalbumine (PS-ova) in de andere test. De detectie van sulfamethazine, sulfamerazine, sulfadimethoxine, sulfadiazine, sulfathiazole, sulfapyridine, sulfachloropyridazine en sulfisoxazole in bufferoplossing werden getest. Hogere antistoffentiters werden bekomen in de ELISA gecoat met TS-ova (TS-ciELISA) in vergelijking met de ELISA gecoat met PS-ova (PS-ciELISA). Anderzijds was de detectie van sulfonamiden gevoeliger in de PS-ciELISA omdat alle geteste sulfonamiden gedetecteerd werden tot de MRL (100 ng/ml). Kruisreacties werden waargenomen voor *p*-aminobenzoëzuur (IC50 >10 µg/ml), maar niet voor diuretica (furosemide, acetazolamide, hydrochlorothiazide, bumetanide), thiamfenicol, florfenicol en lidocaine. Para-aminobenzoëzuur bevat de gemeenschappelijke sulfonamide *p*-aminobenzoylring. Dit wijst er bijgevolg op dat het epitoop dat herkend wordt door de polyklonalen deze *p*-aminobenzoylring bevat. Substitutie van de sulfonamide-groep op de gemeenschappelijke *p*-aminobenzoylring bevordert de binding van de polyklonalen vermits de meeste sulfonamiden beter herkend werden (IC50 <1 µg/ml) dan para-aminobenzoëzuur (IC50 >10 µg/ml).

In een volgende fase werd een extractieprocedure ontwikkeld voor de detectie van sulfonamiden in spier-, nier-, vet en leverweefsels, in beide ELISA's. Sulfachloropyridazine werd als model gebruikt. Een carbonate/bicarbonate buffer (pH 10) werd gekozen als extractiebuffer daar de meeste sulfonamiden zeer goed oplossen in deze buffer. Verschillende homogenisatiemethoden (high-speed mixer (ultraturax) *versus* vortex) werden vergeleken en het effect van kaolin (gehydrateerde aluminium silicaat) werd nagegaan om de achtergrondsignalen in de ELISA's te verminderen. De beste extractieprocedure was deze waarbij gebruikgemaakt werd van een vortex en zonder kaolinbehandeling. Sulfachloropyridazine werd gemakkelijk gedetecteerd aan de MRL in alle weefsels. De beslissingsgrens en het detectievermogen werden bepaald voor sulfachloropyridazine in varkensnieren ($CC\alpha$ = 9.4 ng/ml, $CC\beta$ = 12.9 ng/ml). Aanvaardbare correlaties werden waargenomen tussen de PS-ciELISA en LC-MS/MS voor de analyse van lever en –nierweefsels afkomstig van met sulfachloropyridazine behandelde varkens (r^2 = 0.88 en 0.77 resp.).

Een algemene discussie en conclusies zijn terug te vinden in deel V van deze thesis. De resultaten tonen aan dat het weldegelijk mogelijk is om antistoffen te ontwikkelen specifiek voor een groep antimicrobiële stoffen. Hierbij is het heel belangrijk om een geschikt

immunogeen te vinden voor de inductie van groepsspecifieke antistoffen. Bovendien kunnen enkele richtlijnen neergeschreven worden voor de immunizatie, zoals het afwisselend toedienen van verschillende immunogenen voor het verhogen van de polyklonale antistoffenrespons, en het toedienen van een finale intraveneuze immunizatie ipv intraperitoneaal voor het verhogen van de productie van de hybridomen. Echter, noch het immunogeen noch de immunizatieprocedure kan de productie van geschikte antistoffen garanderen.

Zeer belangrijk bij de selectie van geschikte antistoffen is de test die gebruikt wordt bij deze selectie. De test moet zo goed mogelijk lijken op de screeningstest die men later wil gebruiken. Zowel met de mAb als met de pAb werden voor de penicillines maar ook voor de sulfonamiden, groepsspecifieke testen ontwikkeld. Het is dus mogelijk om een screeningstest te ontwikkelen met groepsspecifieke antistoffen. Hierbij beïnvloedt het testsysteem de gevoeligheid en specificiteit van de antistoffen. In deze thesis bleek de detectie van analyten gebruikmakend van polyklonale antistoffen gevoeliger dan wanneer monoklonalen gebruikt werden.

Teneinde weefsels te kunnen analyseren, moet een geschikte extractieprocedure worden ontwikkeld. Dit werd uitgevoerd voor zowel de penicillines als de sulfonamiden. Het is dus mogelijk om een extractieprocedure te ontwikkelen voor een groep antimicrobiële stoffen. Hierbij zijn de extractiebuffer en de manipulaties van het staal heel belangrijk. Deze laatste moeten bovendien liefst zo eenvoudig mogelijk gehouden worden.

Een methode mag enkel gebruikt worden voor screening indien men kan bewijzen dat de methode voldoet aan de eisen neergeschreven in de Europese richtlijn 2002/657/EC. Voor een kwalitatieve screeningstest wordt gevraagd om het detectievermogen (CCβ), de selectiviteit/specificiteit en de toepasbaarheid, ruwheid en stabiliteit aan te tonen. Wanneer het de bedoeling is om een screeningstest kwantitatief te gebruiken, dan moet de nauwkeurigheid ook bepaald worden. De validatie wordt uitgevoerd met behulp van aangerijkte stalen. Het is echter aangewezen om ook de analyse van praktijkstalen met de screeningstest te vergelijken met de analyse door middel van een referentiemethode. Immers, een methode ontwikkeld met behulp van aangerijkte stalen zal niet noodzakelijk geschikt zijn voor stalen afkomstig van behandelde dieren (praktijkstalen) omdat de interactie tussen het analyt en de weefselmatrix verschillend kan zijn voor aangerijkte stalen in vergelijking met stalen van behandelde dieren. Vergelijkende studies tussen onze ELISA's en een commerciële immunochemische test in geval van penicillines of een gevalideerde chemische analysemethode in geval van

sulfonamiden hebben de geschiktheid aangetoond van onze methoden voor de detectie van deze antimicrobiële stoffen in varkensweefsels.

References

Ahmad A., Ramakrishnan A., McLean M.A., Li D., Rock M.T., Karim A., Breau A.P. (2002) Use of optical biosensor technology to study immunological cross-reactivity between different sulfonamide drugs. Anal. Biochem., 300, 177-184.

Alfredsson G., Ohlsson A. (1998) Stability of sulphonamide drugs in meat during storage. Food Addit. Contam., 15, 302-306.

Allison J.R.D. (1985) Antibiotic residues in milk. British Veterinary Journal, 141, 1, 9-16.

Al-Sam S., Linton A.H., Bennett P.M., Hinton M. (1993) Effects of low concentrations of ampicillin in feed on the intestinal *Escherichia coli* of Chicks. J. Appl. Bacteriol., 75, 108-112.

Anonymous (1990) Council Regulation N° 2377/90 of 26 june 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Commun., N° L224, 1-5.

Anonymous (1991) Food and Drug Regulation, Table III, Division 15, Part B., Canada Gazette Part II, 125, p. 1478-1480.

Anonymous (1992) Commission Regulation N° 675/1992 of 18 March 1992. Off. J. Eur. Communities L73, 19/3/1992, p8.

Anonymous (1995) Ministerieel Besluit van 19 juni 1995 tot de wijziging van het Ministerieel Besluit van 18 december 1973 tot bepaling van de laboratoriumtechnieken voor het opsporen van residuen van stoffen met een kiemgroeiremmende werking. Belgisch Staatsblad, 28.7.1995

Anonymous (1996a) Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products. Off. J. Eur. Commun., L125, 10-32.

Anonymous (1996b) Commission Regulation N° 281/1996 of 14 February 1996. Off. J. Eur. Commun., L37, 15/2/1996, p9.

Anonymous (1997a) Koninklijk Besluit betreffende maatregelen inzake verhandeling van landbouwdieren , ten aanzien van bepaalde stoffen of residu's daarvan met farmacologische werking. Belgisch Staatsblad, 7.10.1997, p 26283-26285.

Anonymous (1997b) The medical impact of the use of antimicrobials in food animals. Report of a WHO meeting, Berlin, Germany, 13-17 October 1997.

Anonymous (1999) Commission Regulation N° 508/1999. Off. J. Eur. Communities L60, 9/3/1999, p 16-52.

Anonymous (2000) Commission Regulation N° 1286/2000 of 19 June 2000. Off. J. Eur. Communities L145, 20/6/2000, p15.

Anonymous (2002) Commission Decision 2002/657/EC of 13 august 2002 implementing Council Directive 96/23/EC concerning the performances of analytical methods and the interpretation of results. Off.J.Eur.Commun., N° L221, 8-34.

Arts C.J.M., Geijp E.M.L, Stark J., Witkamp R.F. (2000) The Premi®test, a broad-spectrum screening test for the detection of antimicrobial compounds in meat, organs and urine. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 186-192.

Assil H.I, Sheth H., Sporns P. (1992) An ELISA for sulfonamide detection using affinity-purified polyclonal antibodies. Food Research International, 25, 343-353.

Avrameas S., Uriel J. (1966) Compt. Rend. Acad. Sci. Paris, 265, 1149.

Babiel R., Maurer E.; Deeg R. (1993) Optimization and validation of methods. In: Methods of immunological analysis. Ed. By Masseyeff R.F., Albert W.H., Staines N.A.; VCH Verslagsgesellschaft mbH, Weinheim, Germany, p.590-591.

Bergstrom C (1998) Design and use of optical biosensor assays in analysis of antimicrobial drug residues in milk. PhD Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Bevill R.F. (1984) Factors influencing the occurrence of drug residues in animal tissues after the use of antimicrobial agents in animal feeds. JAVMA, 185, 1124-1126.

Bienenmann-Ploum M., Korpimäki T., Haasnoot W., Kohen F. (2004) Comparison of multi-sulfonamide biosensor immunoassays. In: Euroresidue V. Conference on residues of veterinary drugs in food. Ed. by R.W. Stephany and A.A. Bergwerff. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 319-324.

Bjurling P., Baxter G.A., Caselunghe M., Jonson C., O'Connor M.C., Persson B., Elliott C.T. (2000) Biosensor assay of sulfadiazine and sulfamethazine residues in pork. Analyst, 125, 1771-1774.

Blanchflower W.J., Hewitt S.A., Kennedy D.G. (1994) Confirmatory assay for the simultaneous detection of five penicillins in muscle, kidney and milk using liquid chromatography-electrospray mass spectrometry. Analyst, 119, 2595-3601.

Blumberg P.M., Strominger J.L. (1974) Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev., 38, 291-335.

Boersma WJA, Claassen E. (1995) Immunization and adjuvant. In: Immunological Recognition of Peptides in Medicine and Biology. Ed. by Zegers N. D., Boersma W.J.A., Claassen E., CRC Press, pp. 169-193.

Boison J.O., (1995) Chemical analysis of ß-lactam antibiotics. Chemical analysis for antibiotics used in agriculture. Ed. by Hisao Oka ea. Arlington (va), AOAC International, pp. 235-306

Boison J.O., Korsrud G.O., Papich M.G., MacNeil J.D., Yates W.D.G. (1992) J. AOAC Int., 75, 974.

Boison J.O.K., Keng L., MacNeil J.D. (1994) Analysis of penicillin G in milk by liquid chromatography J. AOAC Int., 77, 565-570.

Boison J.O., Keng L.J.Y. (1998) Improvement of the multiresidue liquid chromatographic analysis of residues of mono- and dibasic penicillins in bovine and muscle tissues. J. AOAC Int., 81, 1267-1272.

Bonroy K. (2002) A novel approach for the detection of antibiotics using mixed SAMs of thiols on gold. Abstract and posterpresentation, AVS 49th International Symposium, 4-8/11/2002, Denver, USA.

Booman P. (1988) Application of monoclonal antibodies in animal production: a review. Livest. Prod. Sci., 18, 199-215.

Budavari S. (1988) The Merck Index. An encyclopedie of chemicals, drugs, and biologicals. Eleventh edition. Merck & Co, New Jersey, USA, p 8871 – 8929.

Burgat-sacaze V. (1981) Risque d'accidents allergiques dus aux résidus. Rec. Méd. Vét., 157, 187-190.

Bush K., Jacoby, G.A., Medeiros A.A. (1995) A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother., 39, 1211-1233.

Butaye P., Devriese L., Haesebrouck F. (2001) Veterinair gebruikte β-lactam antibiotica : activiteit en resistentie. Vlaams Diergeneeskundig Tijdschrift, 70, 172-179.

Cambell A.M. (1984) Monoclonal antibody technology. Laboratory techniques in biochemistry and molecular biology. Ed. by Burdon B.H., van Knippenberg P.H. Elsevier science publishers, Amsterdam, The Netherlands.

Chaiet I., Wolf F.J. (1964) The properties of streptavidin, a biotin-binding protein produced by *Streptomycetes.* Arch. Biochem. Biophys., 106, 1-5.

Charm E.S., Chi R. (1988) Microbial receptor assay for rapid detection and identification of seven families of antimicrobial drugs in milk: collaborative study. J AOAC, 71, 304-316.

Choi J., Kim C., Choi M.J. (2002) Effects of the competitor on antibody-hapten binding in immunoassay. J. Immunoassay Immunochem., 23, 69-83.

Chowdhury B.A., Lieberman P.L. (1999) Drug allergens, Haptens, and anaphylatoxins. In : Allergens and allergen immunotherapy. Ed. by RF Lockey, SC Bukantz, M. Dekker, second edition, , pp. 273-296.

Cliquet P., Bonroy K., Goddeeris B.M., Cox E. (2004) Penicillin specific antibodies: Monoclonals versus polyclonals in ELISA and in an optical biosensor. Food and Agricultural Immunology, accepted with minor modifications.

Cliquet P., Cox E., Haasnoot W., Schacht E., Goddeeris B.M. (2003a) Generation of group-specific antibodies against sulfonamides. J. Agric. Food Chem., 51, 5835-5842.

Cliquet P., Cox E., Haasnoot W., Schacht E., Goddeeris B.M. (2003b) Extraction procedure for sulfachloropyridazine in porcine tissues and detection in a sulfonamide-specific ELISA. Analytica Chimica Acta, 494, 21-28.

Cliquet P., Cox E., Okerman L., De Wasch K., Goddeeris B.M. (1999) ELISA for the detection of residues of the penicillin group in food of animal origin. Med. Fac. Landbouww. Univ. Gent 64(5b), 507-512.

Cliquet P., Cox E., Van Dorpe C., Schacht E., Goddeeris B.M. (2001) Generation of class-selective monoclonal antibodies against the penicillin group. J. Agric. Food Chem., 49, 3349-3355.

Cliquet P., Goddeeris B.M., Cox E., (submitted) Production of penicillin specific polyclonal antibodies for a class-specific screening ELISA. Journal of Agricultural and Food Chemistry

Cliquet P., Van Dorpe C., Cox E., Goddeeris B.M. (1998) Optimization of an ELISA for the detection of penicillin in food of animal origin. Med.Fac.Landbouww.Univ.Gent 63(4b), 1411-1416.

Cook C.E., Twine M.E., Meyers M., Amerson E., Kepler J.A., Taylor G.F. (1976) Theophylline radioimmunoassay: synthesis of antigen and characterization of antiserum. Res Commun Chem Pathol Pharmacol., 13, 497-505.

Coyle P.V., Wyatt D., McCaughey C., O'Neill H. J. (1992) A simple standardised protocol for the production of monoclonal antibodies against viral and bacterial antigens. J. Immunol. Methods, 15, 81-84.

Crabbe P.M. (2002) Immunochemical screening for sulfamethazine and clostebol acetate residues in urine of food producing animals. Proefschrift voorgelegd tot het verkrijgen van de graad van Doctor in de farmaceutische wetenschappen, Universiteit Gent.

Creighton T.E. (1993) Chapter 8: Interaction with other molecules. In: Proteins. Structures and molecular properties. Ed. by T.E. Creighton, Publisher W.H. Freeman and company, USA, pp. 351-352.

Crooks S.R.H., Baxter G.A., O'Connor M.C., Elliot C.T. (1998) Immunobiosensor – an alternative to enzyme immunoassay screening for residues of two sulfonamides in pigs. The Analyst, 123, 2755-2757.

Crooks S.R.H., Mc Caughey W.J., Elliott C.T., McEvoy J.D., Hewitt S.A. (1996) The production of pig tissue sulphadimidine reference material. Food additive and contaminants, 13, 211-219.

Curvers P. (1993) Immunologische technieken voor het opsporen van antibiotica-residuen in vlees. Proefschrift voorgelegd tot het verkrijgen van een postgraduaat in het diergeneeskundig toezicht over eetwaren van dierlijke oorsprong. Faculteit diergeneeskunde, Universiteit Gent.

De Baere S., Baert K., Croubels S., De Busser J., De Wasch K., De Backer P. (2000) Determination and quantification of sulfadiazine and trimethoprim in swine tissues using liquid chromatography with ultraviolet and mass spectrometric detection. Analyst, 125, 409-415.

De Baere S., Cherlet M., Baert K., De Backer P. (2002) Quantitative analysis of amoxycillin and its major metabolites in animal tissues by liquid chromatography combined with electrospray ionisation tandem mass spectrometry. Analytical Chemistry, 74, 1393-1401.

de Haan, P., de Jonge A.J.R., Verbrugge T., Boorsma D.M. (1985) Three epitope-specific monoclonal antibodies against the hapten penicillin. Int. Archs. Allergy Immun., 76, 42-46.

De Leuw P., Kapa G., Petz M. (1997) Induction and characterization of multianalyte antibodies against penicillins in egg yolk. J. AOAC Int. 80 (6), 1220-1228.

Delagneau J.F., Masseyeff R. (1990) Criteria for the selection of a solid phase to be used in immunoassays. Ann. Biol. Clin. Paris, 48, 467-471.

Dewdney J. M., Maes L., Raynaud J.P., Blanc F., Scheid J.P., Jackson T., Lens S., Verschueren C. (1991) Risk assessment of antibiotic residues of β-lactams and macrolides in food products with regard to their immuno-allergis potential. Fd. Chem. Toxic., 29, 477-483.

Dietrich R., Usleber E., Märtlbauer E. (1998) The potential of monoclonal antibodies against ampicillin for the preparation of a multi-immunoaffinity chromatography for penicillins. Analyst, 123, 2749-2754.

Divers T.J. (1996) Penicillin therapy in bovine practice. The Compendium, 18, 703-709

Dixon-Holland D.E, Katz S.E. (1988) Competitive direct enzyme-linked immunosorbent assay for the detection of sulfamethazine residues in swine urine and muscle tissue. J. Assoc. Off. Anal. Chem., 71, 1137-1140.

Dupont H.L., Steele J.H. (1987) Use of antimicrobial agents in animal feeds: implications for human health. Review. Rev Infect Dis., 9, 447-460.

Eisen H.N., Siskind G.W. (1964) Variations in affinities of antibodies during the immune response. Biochemistry, 3, 996-1008.

Elliot C.T. (2001) Biacore's SPR technology in routine analysis. BIA Journal, 2, 14-15.

Elliott C.T., Baxter G.A., Crooks S.R.H., McCaughey W.J. (1999) The development of a rapid immunobiosensor screening method for the detection of residues of sulfadiazine. Food and Agricultural Immunology, 11, 19-27.

Ellman G. L. (1959) Tissue sulfhydryl groups. Arch. Biochem. Biophys., 82, 70-77.

Everest D.J., Everest S.J., Jackman R. (1993) Spectrometric assay for ß-lactam residues in kidney tissues. Analytica Chimica Acta, 275, 249-252.

Fink A.L., Behner K.M., Tan A.K. (1987) Kinetic and structural characterization of reversibly inactivated β-lactamase. Biochemistry, 26, 4248-4258.

Fleeker J.R., Lovett L.J. (1985) Enzyme Immunoassay for screening sulfamethazine residues in swine blood. J. Assoc. Off. Anal. Chem., 68, 172-174.

Franco D.A., Webb J., Taylor C.E. (1990) Antibiotic and sulfonamide residues in meat: implication for human health. Journal of Food Protection., 53, 178-185.

Garden S.W., Sporns P. (1994) Development and evaluation of an enzyme immunoassay for sulfamerazine in milk. J. Agric. Food Chem., 42, 1379-1391.

Gaudin V., Marris P. (2000) Development of a biosensor-based immunoassay for the screening of penicillins residues in milk. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 449-454.

Grubelnik A., Padeste C., Tiefnauer L. (2001) Highly sensitive enzyme immunoassays for the detection of β-lactam antibiotics. Food and Agricultural Immunology, 3, 161-169.

Grunwald L., Petz M. (2003) Food processing effects on residues: penicillins in milk and yoghurt. Analytica Chimica Acta, 483, 73-79.

Guggisberg D, Mooser A.E., Koch H. (1992) Methods for the determination of sulphonamides in meat. J. Chromatogr. 624, 425-437.

Guillot J.F. (1989). Apparition et évolution de la résistance bactérienne aux antibiotiques. Annales de recherches véterinaires, 20, 3-16.

Gunnarsson K. (1998) From new technology to standard technique – in less than ten years. BIA Journal, 1, 5-7.

Haasnoot W., Bienenmann-Ploum M., Kohen F. (2003) Biosensor immunoassay for the detection of eight sulfonamides in chicken serum. Analytica Chimica Acta., 483, 171-180.

Haasnoot W., Cazemier G., Du Pré J., Kemmers-Voncken A., Bienenmann-Ploum M., Verheijen R. (2000b) Sulphonamide antibodies: from specific polyclonals to generic monoclonals. Food and Agricultural Immunology, 12, 15-30

Haasnoot W., Du Pré J., Cazemier G., Kemmers-Voncken A., Verheijen R., Jansen B.J.M. (2000a) Monoclonal antibodies against a sulfathiazole derivative for the immunochemical detection of sulfonamides. Food and Agricultural Immunology, 12, 127-138.

Haasnoot W., Kohen F., Du Pré J., Cazemier G., Kemmers-Voncken A., Bienenmann-Ploum M., Verheijen R. (2000c) Application of generic monoclonal antibodies against sulfonamides in optical biosensors. In: Euroresidue IV. Conference on residues of veterinary drugs in food.

Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 501-505.

Haasnoot W., Korsrud G.O., Cazemier G., Maneval F., Keukens H., Nouws J. (1996) Application of an enzyme immunoassay for the determination of sulphamethazine (sulphadimidine) residues in swine urine and plasma and their use as predictors of the level in edible tissue. Food additive and contaminants, 13, 811-822.

Haasnoot W., Loomans E.E.M.G., Cazemier G., Dietrich R., Verheijen R. Bergwerff A.A., Stephany R.W. (2002) Direct versus competitive biosensor immunoassays for the detection of (dihydro)streptomycin residues in milk. Food and Agricultural Immunology, 14, 15-27.

Haasnoot W., Verheijen R. (2001) A direct (non-competitive) immunoassay for gentamicin residues with an optical biosensor. Food and Agricultural Immunology, 13, 131-134.

Haesebrouck F., Devriese L. (1994) Verworven antibiotica-resistentie en orale antibiotica medicatie bij landbouwhuisdieren. Vlaams Diergeneeskundig Tijdschrift, 63, 3-6.

Harlow E. and Lane D., Eds. (1988) Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, New York, US. pp. 139-243

Hermanson G.T., Ed. (1996) Bioconjugate Techniques. Academic Press Inc, New York, US., pp 118.

Hou J.P., Poole J.W. (1971) Beta-lactam antibiotics: their physicochemical properties and biological activities in relation to structure. J. Pharm. Sci., 60, 503-532.

Huth S.P., Warholic P.S., Devou J.M., Chaney L.K., Clark G.H. (2002) Parallux beta-lactam: a capillary-based fluorescent immunoassay for the determination of penicillin-G, ampicillin, amoxicillin, cloxacillin, cephapirin, and ceftiofur in bovine milk. J. AOAC. Int., 85, 355-364.

Ito Y., Oka H., Ikai Y., Matsumoto H., Miyazaki Y., Nagase H. (2000) Application of ion-exchange cartridge clean-up in food analysis. Simultaneous determination of sulphonamide antibacterials in animal liver and kidney using high-performance liquid chromatography with ultraviolet and mass spectrometric detection. J Chromatogr A. 898, 95-102.

Janosi A., Maghuin-Rogister G., Degelaen J. (2000) A sensitive screening method for the multi-analyte determination of β-lactam antibiotic residues in swine tissue. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 639-644.

Jemmerson R. (1995) Effects on immunological recognition of peptides. In : Synthetic peptides in Medecine and Biology, (ED), CRC Press Inc., pp. 213-225.

Jonsson U, Fagerstam L, Ivarsson B, Johnsson B, Karlsson R, Lundh K, Lofas S, Persson B, Roos H, Ronnberg I (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. Biotechniques, 11, 620-627.

Kachab E.H., Wu W.Y, Chapman C.B. (1992) The development of an enzyme-linked immunosorbent assay (ELISA) for cephalexin. Journal of Immunol. Methods, 147, 33-41.

Kanavanagh F., (1989) Theory and practise of microbiological assaying for antibiotics, J AOAC, 72, 6-10.

Karlsson R., Falt A. (1997) Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. J. Immunol. Methods, 200, 121-133.

Karlsson R., Kullman-Magnusson M, Hamalainen M.D., Remaeus A., Andersson K., Borg P., Gyzander E., Deinum J. (2000) Biosensor analysis of drug-target interactions: direct and competitive binding assays for investigation of the interactions between thrombin and thrombin inhibitors. Analytical Biochemistry, 278, 1-13.

Karlsson R., Stahlberg R. (1995) Surface plasmon resonance detection and multispot sensing for direct monitoring of interactions involving low-molecular-weight analytes and for determination of low affinities. Analytical Biochemistry, 228, 274-280.

Karsten U., Stolley P., Walther I., Papsdorf G., Weber S., Conrad K., Pasternak L., Kopp J. (1988) Direct comparison of electric field-mediated and PEG-mediated cell fusion for the generation of antibody producing hybridomas. Hybridoma, 7, 627-633.

Katsutani N., Shionoya H. (1993) Immunogenicity of various ß-lactam antibiotic-protein conjugates and cross-reactivity of the antibodies produced in Guinea Pig. Int. Arch. Allergy Immunol. 100, 128-134.

Kheirolomoon A., Kazemi-Vaysari A., Ardjmand M., Baradar-Khoshfetrat A. (1999) The combined effects of pH and temperature on penicillin G decomposition and its stability modeling. Process Biochemistry, 35, 205-211.

Kirkley J.E., Goldstein A.L., Naylor P.H. (2001) Effect of peptide carrier coupling on peptide-specific immune responses. Immunobiology, 203, 601-615.

Kitagawa T., Gotoh Y., Uchihara K., Kohri Y., Kimove T., Fujiwara K., Ohtani W. (1988) Sensitive immunoassay of cephalexin residues in milk, hen tissues and eggs. J. Assoc. Off. Anal. Chem., 71, 915-920.

Kitagawa T., Kanamura T., Wakamatsu H., Kato H., Yano S., Asanuma Y. (1978) A new method for preparation of an antiserum to penicillin and its application for novel enzyme immunoassay of penicillin. Journal of Biochemistry, 84, 491-494.

Knowles S., Shapiro L., Shear N.H. (2001) Should celecoxib be contraindicated in patients who are allergic to sulfonamides? Revisiting the meaning of ‘sulfa’ allergy. Drug Saf. 24, 239-247.

Koenen-Dierick K., Van Hoof J. (1988) Mislukkingen in de droge worstbereiding door aanwezigheid van penicilline- en tetracycline-residuen in vlees. Vlaams Dierg. Tdschr., 57, 109-112.

Kohen F., Gayer B., Amir-Zaltsman Y., O'Keeffe M. (2000) Generation of an anti-idiotypic antibody as a surrogate ligand for sulfamethazine in immunoassay procedures. Food and Agric. Immunology, 12, 193-201.

Köhler G., Milstein C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256, 495-497.

Korpimäki T., Rosenberg J., Virtanen P., Karskela T., Lamminmäki U., Tuomola M., Vehniäinen M., Saviranta P. (2002) Improving broad specificity hapten recognition with protein engineering. J. Agric Food Chem, 50, 4194-4201.

Korpimäki T., Rosenberg J., Virtanen P., Lamminmäki U., Tuomola M., Saviranta P. (2003) Further improvement of broad specificity hapten recognition with protein engineering. Protein Engineering, 16, 37-46.

Korsrud G.O., Papich M.G., Fesser A.C.E., Salisbury C.D.C., Macneil J.D. (1996) Residue depletion in tissues and fluids from swine fed sulfamethazine, chlortetracycline and penicillin G in combination. Food Addit Contam, 13, 287-292.

Kramer K. (2002) Synthesis of a group-selective antibody library against haptens. Journal of Immunol. Methods, 266, 209-220.

Lee N., Holtzapple C.K., Muldoon M.T., Deshpande S.S., Stanker L.H. (2001) Immunochemical approaches to the detection of sulfathiazole in animal tissues. Food and Agricultural Immunology, 13, 5-17.

Li J.S., Li X.W., Yuan J.X., and Wang X. (2000) Determination of sulfonamides in swine meat by immunoaffinity chromatography. J. Assoc. Off. Anal. Chem., 83, 830-836.

Long A.R., Hsieh L.C., Malbrough M.S., Short C.R., Barker S.A. (1990). Multiresidue method for the determination of sulfonamides in pork tissue. Journal of agricultural and food chemistry, 38, 423-426.

Lynas L., Currie D., McEvoy J.D.G. (2000) Post screening methods for the detection of β-lactam residues in pigs. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 728-732.

Markey F. (1998) Biacore 3000- primed to perfection. BIA journal, 2, 4-7.

Märtlbauer E., Ed. (1993) Enzymtests für antimikrobiell wirksame stoffe. Ferdinand Enke Verlag, Stuttgart, Germany., pp 28.

Mayorga C., Obispo T., Jimeno L., Blanca M., Moscoso del Prado J., Carreira J., Garcia J. J., Juarez C. (1995) Epitope mapping of β-lactam antibiotics with the use of monoclonal antibodies. Toxicology, 97, 225-234.

McCracken R.J., Spence D.E., Kennedy D.G. (2000) Comparison of extraction techniques for the recovery of veterinary drug residues from animal tissues. Food Addit Contam., 17, 907-914.

McEvoy J.D.G. (2002) Contamination of animal feedingstuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. Analytica Chimica Acta, 473, 3-26.

McEvoy J.D.G., Higgins H.C., Mayne C.S., Kennedy D.G. (2000) Transfer of sulfadiazine from contaminated dairy feed to cows' milk. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 753-757.

McGrath T., Ferguson J., Haughey S., Baxter A. (2004) Rapid multi-sulfonamide detection in procine muscle using SPR biosensor technology. In: Euroresidue V. Conference on residues of veterinary drugs in food. Ed. by R.W. Stephany and A.A. Bergwerff. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 665-670.

Medina M.B., Poole D.J., Anderson M.R. (1998) A screening method for beta-lactams in tissues hydrolyzed with penicillinase I and lactamase II. J AOAC Int. 81, 963-972.

Meekins C.V., Sullivan T.J., Gruchalla R.S. (1994) Immunochemical analysis of sulfonamide drug allergy: identification of sulfamethoxazole-substituted human serum proteins. J. Allergy Clin. Immunol., 94, 1017-1024.

Milhaud G., Person J.M. (1981) Evaluation de la toxicité des résidus d' antibiotiques dans le lait. Réc. Med. Vet., 157, 9-16.

Mirza I.H., Wilkin T.J., Cantarini M., Moore K. (1987) A comparison of spleen and lymph node cells as fusion partners for raising of monoclonal antibodies after different routes of immunisation. J. Immunol. Methods, 105, 235-243.

Mitchell J.M., Yee A.J., McNab W.B., Griffiths M.W., McEwen S.A. (1999) Validation of the LacTek test applied to spiked extracts of tissues samples: determination of performance characteristics. J AOAC Int., 82, 79-84.

Moats W.A., (1990) Determination of penicilline G in milk by high performance liquid chromatography with automated liquid chromatography clean-up. J. Chrom., 507, 177-185.

Mosmann T.R., Gallatin M., Langenecker B.M. (1980) Alteration of apparent specificity of monoclonal (hybridoma) antibodies recognizing polymorphic histocompatibility and blood group determinants. J. Immunol. 125, 1152-1156.

Mourot D., Loussouarn S. (1981) Sensibilité des ferments lactiques aux antibiotiques utilisés en Médecine Vétérinaire. Réc. Méd. Vét., 157, 175-177.

Muldoon M.T., Font I.A., Beier R.C., Holtzapple C.K., Young C.R., Stanker L.H. (1999) Development of a cross-reactive monoclonal antibody to sulfonamide antibiotics: evidence for structural conformation-selective hapten recognition. Food and Agricultural Immunology, 11, 117-134.

Muldoon M.T., Holtzapple C.K., Deshpande S.S., Beier R.C., Stanker L.H. (2000a) Development of a monoclonal antibody-based cELISA for the analysis of sulfadimethoxine.

1. Development and characterization of monoclonal antibodies and molecular modeling studies of antibody recognition. J. Agric. Food Chem., 48, 537-544.

Muldoon M.T., Buckley S.A., Deshpande S.S., Holtzapple C.K., Beier R.C., Stanker L.H. (2000b) Development of a monoclonal antibody-based cELISA for the analysis of sulfadimethoxine. 2. Evaluation of rapid extraction methods and implications for the analysis of incurred residues in chicken liver tissue. J. Agric. Food Chem., 48, 545-550.

Murphy B.E.P. (1980) Pitfalls in the assay of cortisol. J. Immunoassay, 1, 413- 434.

Nagakura N., Souma S., Shimizu T., Yanagihara Y. (1991) Anti-ampicillin monoclonal antibodies and their cross-reactivities to various β-lactams, Journal of antimicrobial Chemotherapy, 28, 357-368.

Nakane P.K., Pierce G.B. (1966) Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem., 14, 929.

Nicklas W. (1992) Aluminum saltz. Research in Immunology, 143, 489-494.

Nolan ML., Dooley M., Nugent A., O'Keeffe M. (2000) Antibiotic residue testing in kidney tissue: comparison of a modified one-plate test with the EC four-plate test and the Charm II test. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 781-785.

Nouws J.F.M., Mevius D.J., Vree T.B., Baakman B.S., Degen M. (1988) Pharmacokinetics, metabolism, and renal clearance of sulfadiazine, sulfamerazine and sulfamethazine and their N_4-acetyl and hydroxy metabolites in calves and cows. Am. J. Vet. Res., 49, 1059-1065.

Nouws J.F.M., Mevius D.J., Vree T.B., Degen M. (1989) Pharmacokinetics and renal clearance of sulphadimidine, sulphamerazine and sulphadiazine and their N_4-acetyl and hydroxy metabolites in pigs. The Veterinary Quarterly, 11, 78-86.

Okerman L. (1995) Vergelijking van methoden toegepast bij het screenen van slachtdieren op aanwezigheid van antibiotica-residuen. Proefschrift postgraduaat, Diergeneeskundig toezicht op eetwaren. Faculteit diergeneeskunde, Universiteit Gent.

Okerman L., De Wasch K., De Brabander H., Abrams R., Van Hoof J., Cornelis M., Laurier L. (1999). Oude en nieuwe opsporingstechnieken voor antibioticaresiduen in het kader van de huidige Belgische en Europese wetgeving. Vlaams Diergeneeskundig Tijdschrift, 68, 216-223.

Okerman L., De Wasch K., Van Hoof J. (1998a) Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix. Analyst, 123, 2361-2365.

Okerman L., De Wasch K., Van Hoof J., Smedts W. (2003) Simultaneous determination of different antibiotic residues in bovine and in porcine kidneys by solid-phase fluorescence immunoassays. J AOAC Int., 86, 236-240.

Okerman L., Debeuckelaere W., Van Hoof J. (1998b) Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets. J AOAC Int., 81, 51-56.

Ormerod A.D., Reid T.M.S., Main R.A. (1987) Penicillin in milk-its importance in urtucaria. Clinical Allergy, 17, 229-234.

Oshiro B.T. (1999) The semisynthetic penicillins. Prim. Care Update, 6, 56-60.

Panayotou G. (1998) Surface Plasmon Resonance. Encyclopedia of immunology. Ed. by Delves P.J. and Roitt I.M. Academic Press Limited, London, UK. pp. 2247-2250.

Paraf A., Peltre G., Eds. (1991) Immunoassays in Food and Agriculture. Kluwer Academic Publishers, London, UK, pp. 294-311.

Phillips I., Casewell M., Cox T., De Groot B., Friis C., Jones R., Nightingale C., Preston R., Waddell J. (2004) Does the use of antibiotics in food animal pose a risk to human health? A critical review of published data. J. Antimicrobial Chemotherapy, 53, 28-52.

Poirier L.A., Doerge D.R., Gaylor D.W., Miller M.A., Lorentzen R.J., Casciano D.A., Kadlubar F.F, Schwetz B.A. (1999) An FDA review of sulfamethazine toxicity. Regulatory Toxicology and Pharmacology, 30, 217-222.

Raynor B.D. (1997) Penicillin and ampicillin. Prim Care Update, 4, 147-152.

Rehm W.F., Teelmann K., Weidekamm E. (1986) General aspects of metabolism, residues, and toxicology of sulfonamide and dihydrofolate reductase inhibitiors. In: Drug residues in animals. Ed. by André G. Rico., Academic Press, Orlando, USA, pp. 65-79.

Reybroeck W., Ooghe S. (2003) Results of a multilaboratory trial regarding rapid tests for the control of milk on β-lactam antibiotics. In: Strategies for safe food: analytical, industrial and legal aspects: challenges in organisation and communication. Proceedings of the Euro Food Chem XII Conference. Ed. by Eklund T., De Brabander H., Daeseleire E., Dirinck I., Ooghe W. KVCV, Heverlee, Belgium. pp. 523-526.

Robinson G.A. (1993) Optical immunosensors. Methods of immunological analysis. Ed. by Masseyeff R.F., Albert W.H. and Staines N.A., Verlagsgesellschaft mbH, Weinheim, Germany. pp. 372 – 387.

Rohner P., Schällibaum M., Nicolet J.J. (1985) Detection of penicillin G and its benzylpenicilloyl (BPO)-derivatives in cow milk and milk serum by means of an ELISA. Journal of Food Protection, 48, 59-62.

Rubenstein K.E., Schneider R.S., Ullman E.F. (1972) "Homogeneous" enzyme immunoassay. A new immunochemical technique. Biochem. Biophys. Res. Commun., 47, 846-851.

Saunal H., Karlsson R., Van Regenmortel M.H.V. (1997) Antibody affinity measurements. In: Immunochemistry 2: a practical approach. Ed. By Johnstone A.P. and Turner M.W., IRL Press, Oxford, pp. 1-51.

Schmidt P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olsen B.J., Klenk D.C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem., 150, 76-85.

Schmidt Von A. (1983) Chloramphenicolrückstände in Lebensmitteln tierischer Herkunft als potentielle Ursache der aplastischen Anämie des Menschen. Dtsch. Tierärztl. Wschr., 90, 201-248.

Schwarz S., Chaslus-Dancla E. (2001) Use of antimicrobials in veterinary medicine and mechanisms of resistance. Veterinary research, 32, 201-225.

Shearan P, O'Keeffe M. (1994) Novel approach to the "on-site" testing for sulfamethazine in pork carcasses. Analyst, 119, 2761-2764.

Sheth H.B., Sporns P. (1990) Enzyme Immunoassay for Screening of sulfathiazole in Honey. J. Assoc. Off. Anal. Chem., 73, 871-874.

Sheth H.B., Sporns P. (1991) Development of a single ELISA for detection of sulfonamides. J. Agric. Food Chem., 39, 1696-1700.

Sheth H.B., Yaylayan V.A., Low N.H., Stiles M.E., Sporns P. (1990) Reaction of reducing sugars with sulfathiazole and importance of this reaction to sulfonamide residue analysis using chromatographic, colorimetric, microbial, or ELISA methods. J. Agric. Food Chem., 38, 1125-1130.

Sköld O. (2001) Resistance to trimethoprim and sulfonamides. Vet Res, 32, 261-273.

Smit L.A., Haagsma N., Ruiter A. (1999) Binding of sulphadimidine to glucose in sausage. European food research and technology, 209, 201-204.

Spinks C.A., Schut C.G., Wyatt G.M., Morgan M.R. (2001) Development of an ELISA for sulfachloropyridazine and investigation of matrix effects from different sample extraction procedures. Food Addit. Contam., 18, 11-18.

Spinks C.A., Wyatt G.M., Morgan M.R.A. (1999) Molecular modeling of hapten structure and relevance to broad spectrum specificity immunoassay for sulfonamide antibiotics. Bioconjugate Chemistry, 10, 583-588.

Spratt B.G. (1983) Penicillin-binding proteins and the future of β-lactam antibiotics. Journal of General Microbiology, 129, 1247-1260.

Steinitz M., Izak G., Cohen S., Ehrenfeld M., Flechner I. (1980) Continuous production of monoclonal rheumatoid factor by EBV-transformed lymphocytes. Nature, 287, 443-445.

Sternesjo A., Mellgren C., Bjorck L. (1995) Determination of sulfadiazine residues in milk by surface plasmon resonance-based biosensor assay. Anal. Biochem. 226, 175-181.

Stryer L. (1988) Molecular Immunology. In: Biochemistry. Ed. by Stryer L. Freeman W.H. and company, New York, USA, pp. 889-920.

Tanford C. (1978) The hydrophobic effect and the organization of living matter. Science, 200, 1021-1028.

Thomas G.K., Roderick G.M., Anstis P.W. (1997) Stability of sulfonamide antibiotics in spiked pig liver tissue during frozen storage. J AOAC Int., 80, 988-995.

Tijssen P. (1985) Practice and theory of enzyme immunoassays. Laboratory techniques in biochemistry and molecular biology. Ed. by Burdon B.H. and van Knippenberg P.H., Elsevier science publishers, Amsterdam, The Netherlands.

Tilles S.A. (2001) Practical issues in the management of hypersensitivity reactions: sulfonamides. Southern Medical Journal, 94, 817-824.

Trepanier L.A. (1999) Delayed hypersensitivity reactions to sulphonamides: syndromes, pathogenesis and management. Veterinary Dermatology, 10, 241-248.

Usleber E., Litz S., Märtlbauer E. (1998) Production and characterization of group-specific antibodies against penicillin antibiotics. Food and Agricultural Immunology, 10, 317-324.

Usleber E., Lorber M., Straka M., Terplan G., Märtlbauer E. (1994) Enzyme immunoassay for the detection of isoxazolyl penicillin antibiotics in milk. Analyst, 119, 2765-2768.

Usleber E., Strasser A., Dietrich R., Schneider E., Bürk C., Märtlbauer E. (2000) Immunochemical methods for penicillin antibiotics. In: Euroresidue IV. Conference on residues of veterinary drugs in food. Ed. by Van Ginkel L. A. and Ruiter A. National Institute of Public Health and Environment (RIVM), Bildhoven, The Netherlands, pp. 1085-1089.

van de Water C. (1990) Development of immunochemical procedures for the analysis of chloramphenicol residues in food of animal origin. PhD thesis, University of Utrecht, The Netherlands, pp. 51-53.

Van den Bogaard A.E., Stobberingh E.E. (1999) Antibiotic usage in animals: impact on bacterial resistance and public health. Drugs, 58, 589-607.

Van den Broeck W., Cox E., Goddeeris B.M. (1999) Induction of immune responses in pigs following orale administration of purified F4 fimbriae. Vaccine, 17, 2020-2029.

van Duijn G., Langedijk J.P., de Boer M., Tager J.M. (1989) High yields of specific hybridomas obtained by electrofusion of murine lymphocytes immunised in vivo or in vitro. Exp. Cell Res., 183, 463-472.

Van Eechout N, Perez J.C., Van Peteghem C. (2000) Determination of eight sulfonamides in bovine kidney by liquid chromatography/ tandem mass spectrometry with on-line extraction and sample clean-up. Rapid Commun Mass Spectrom. 14, 2331-2338.

Van Oss C.J. (1994) Nature of specific ligand-receptor bonds, in particular the antigen-antibody bond. In: Immunochemistry. Ed. by Van Oss C.J. and Van Regenmortel M.H.V., Publisher M. Dekker, New York, US, pp. 581-615

Van Pethegem C., Daeseleire E. (2003) Drug residue analysis in food and feed: state-of-the-art for growth promotors. In: Strategies for safe food: analytical, industrial and legal aspects: challenges in organisation and communication. Proceedings of the Euro Food Chem XII Conference. Ed. by Eklund T., De Brabander H., Daeseleire E., Dirinck I., Ooghe W. KVCV, Heverlee, Belgium. pp. 379-385.

Van Regenmortel M.H.V., Azimzadeh A. (1994) Determination of antibody affinity. In: Immunochemistry. Ed. by Van Oss C.J. and Van Regenmortel M.H.V., Publisher M. Dekker, New York, US, pp. 805-828.

Van Regenmortel M.H.V., Briand J.P., Müller S., Plaué S. (1988) Synthetic polypeptides as antigen: Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier. New York, US. pp. 95-127.

Van Weeman B.K., Schuurs A.H.W.M. (1975) The influence of heterologous combinations of antiserum and enzyme-labeled estrogen on the characteristics of estrogen enzyme-immunoassays. Immunochemistry, 12, 667-670.

Wiese B., Martin K. (1989a) Basic extraction studies of benzylpenicillin and its determination by liquid chromatography with pre-column derivatisation. J. Pharm. Biomed. Anal., 7, 67-78.

Wiese B., Martin K. (1989b) Determination of benzylpenicillin in plasma and lymph at the ng ml-1 level by reversed-phase liquid chromatography in combination with digital subtraction chromatography technique. J. Pharm. Biomed. Anal., 7, 107-118.

Yarmush M.L., Gates F.T., Weisfogel D.R., Kindt T.J. (1980) Identification and characterization of rabbit-mouse hybridomas secreting rabbit immunoglobulin chains. Proc. Natl. Acad. Sci. USA, 77, 2899-903.

Zurawski V.R., Spedden S.E., Black P.H., Haber E. (1978) Clones of human lymphoblastoid cell lines producing antibody to tetanus toxoid. Curr. Top. Microb. Immunol., 81, 152-155.

Curriculum vitae

Patricia Cliquet werd geboren op 22 juni 1974 te Halle. In 1992 beëindigde zij haar secundaire opleiding, richting Wiskunde, aan het Sint-Niklaasinstituut te Anderlecht. Vervolgens begon ze aan de studies Bioingenieur aan de Vrije Universiteit Brussel, waar ze in 1997 het diploma Bioingenieur in de Cel- en Gentechnologie behaalde met onderscheiding. Onmiddellijk daarna startte ze aan het Laboratorium voor Immunologie van de Huisdieren. Gedurende zes jaar voerde zij onderzoek naar de ontwikkeling van immunochemische technieken voor de detectie van penicillines en sulfonamiden in vleeswaren. Dit onderzoek werd uitgevoerd onder begeleiding van Prof. Dr. E. Cox en Prof. Dr. B. Goddeeris en leidde tot dit proefschrift. Tevens behaalde ze in 2003 het getuigschrift voor de doctoraatsopleiding in de diergeneeskundige wetenschappen. Patricia is auteur en co-auteur van zes wetenschappelijke publicaties.

Publications

Cliquet P., Bonroy K., Goddeeris B.M., Cox E. (2004) Penicillin specific antibodies: Monoclonals versus polyclonals in ELISA and in an optical biosensor. Food and Agricultural Immunology, accepted with minor modifications.

Cliquet P., Cox E., Haasnoot W., Schacht E., Goddeeris B.M. (2003b) Extraction procedure for sulfachloropyridazine in porcine tissues and detection in a sulfonamide-specific ELISA. Analytica Chimica Acta, 494, 21-28.

Cliquet P., Cox E., Haasnoot W., Schacht E., Goddeeris B.M. (2003a) Generation of group-specific antibodies against sulfonamides. J. Agric. Food Chem., 51, 5835-5842.

Cliquet P., Cox E., Van Dorpe C., Schacht E., Goddeeris B.M. (2001) Generation of class-selective monoclonal antibodies against the penicillin group. J. Agric. Food Chem., 49, 3349-3355.

Cliquet P., Goddeeris B.M., Cox E., (submitted) Production of penicillin specific polyclonal antibodies for a class-specific screening ELISA. Journal of Agricultural and Food Chemistry

Schmidt C., Brijs L., Cliquet P. and De Baetselier P. (1998) Increased IL-12 P40 homodimer secretion by spleen cells during in vivo growth of the BW-19 T cell hybridoma accompanies suppression of natural immunity. Int-J-Cancer. Jul 29; 77 (3): 460-466.

Proceedings in national and international conferences

P. Cliquet, E. Cox, C. Van Dorpe, B. Goddeeris (1998) Optimization of an ELISA for the detection of penicillins in food of animal origin. 12th Forum For Applied Biotechnology 24-25/ 09/1998 Brugge, Med. Fac. Landbouww. Univ. Gent 63(4b) 1411-1416.

P. Cliquet, E. Cox, C. Van Dorpe, B. Goddeeris (1999) ELISA for the detection of residues of the penicillin group in food of animal origin. 13th Forum For Applied Biotechnology 22-23/ 09/1999 Gent, Med. Fac. Landbouww. Univ. Gent 64(5b) 507-512.

P. Cliquet, E. Cox, B. Goddeeris (2000) Comparison of an ELISA and ELIFA for the detection of penicillins in meat products. Euroresidue IV. Conference on residues of veterinary drugs in food, Ed. LA. Van Ginkel & A. Ruiter, National institute of public health and environment (RIVM), Bilthoven, Nederland, pp291-296.

P. Cliquet, E. Cox, B. Goddeeris (2003) Immunochemical detection of penicillins in ELISA and in an optical biosensor Conference. Proceedings of the Euro Food Chem XII conference ”Strategies for safe food”. Ed. Eklund T., De Brabander H., Daeseleire E., Dirinck I. and W. Ooghe, KVCV, Heverlee, pp. 425-428.

Lectures, poster presentations and abstracts on national and international conferences

P. Cliquet, E. Cox, C. Van Dorpe, B. Goddeeris (Lecture) Optimization of an ELISA for the detection of penicillins in food of animal origin. 12th Forum For Applied Biotechnology 24-25/ 09/1998 Brugge

P. Cliquet, E. Cox, C. Van Dorpe, B. Goddeeris (Lecture) ELISA for the detection of residues of the penicillin group in food of animal origin. 13th Forum For Applied Biotechnology 22-23/ 09/1999 Gent

P. Cliquet, E. Cox, B. Goddeeris (Poster) Comparison of an ELISA and ELIFA for the detection of penicillins in meat products Euroresidue IV. Conference on residues of veterinary drugs in food, 8-10/05/2000, Veldhoven, The Netherlands

P. Cliquet, E. Cox, C. W. Haasnoot, E. Schacht and B. M. Goddeeris (Poster & abstract) ELISA for the detection of sulfonamides in meat products presentation. 4th international symposium on hormone and veterinary drug residue analysis, 4-7/6/2002, Antwerpen

P. Cliquet (Lecture) Immunodetection of antibiotic residues by ELISA and biosensor. Interuniversity Programm Molecular Biology, KUL, 2 mei 2003, Heverlee

P. Cliquet, E. Cox, B. Goddeeris (Poster) Immunochemical detection of penicillins in ELISA and in an optical biosensor. Conference Euro Food Chem XII, 24-26/9/2003, Brugge

K. Bonroy, F. Frederix, P. Cliquet, G. Reekmans, W. Laureyn, A. Campitelli, G. Borghs, E. Cox, P. Declerck (Poster & abstract) A novel approach for the detection of antibiotics using mixed SAMs of thiols on gold. AVS 49th international symposium, 4-8/11/2002, Denver, USA